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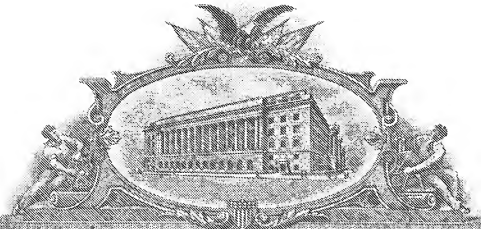

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
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Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

PROCESS OF MAKING PIXYLATING REAGENT AND DERIVATIVES

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Drawing(s) *Number of Sheets* ☐ Other (specify)

☐ Application Date Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE

Date 02/10/2004

REGISTRATION NO. 44,427 *(if appropriate)*

Docket Number: DVCM0002US.L2

TYPED or PRINTED NAME Matthew Grumbling

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Docket Number DVCM0002US.L2

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Number 2 of 2

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PROCESS OF MAKING PIXYLATING REAGENT AND DERIVATIVES

FIELD OF THE INVENTION

The present invention is directed to the field of manufacture of reagents, nucleoside derivatives, nucleoside amidites and oligonucleotide derivatives thereof, as well as methods of using said pixylating reagents and derivatives.

BACKGROUND OF THE INVENTION

Oligomeric compounds having the ability to specifically bind natural and synthetic polynucleotides have found numerous uses in analytical methods for detection, identification, and quantification of polynucleotides, as primers and probes for amplifying genes and gene products (e.g. the polymerase chain reaction, PCR), in target validation studies, and most recently, as therapeutics. Oligomeric compounds such as oligonucleotide DNA and RNA have been used successfully to detect natural polynucleotides and polynucleotide products on so-called biochips. Oligomeric compounds can also be used as primers and probes for taq-polymerase in PCR. Various oligonucleotide compounds and derivatives thereof have been successfully employed in gene-silencing, both *in vitro* and *in vivo*. Such oligonucleotide compounds and their derivatives include so-called antisense compounds – oligomers capable of specifically binding a gene or gene product, and either directly or indirectly effecting silencing of the gene.

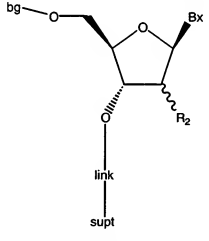
Antisense therapeutics have shown great promise. Antisense therapeutics modulate protein activities by attenuating the concentration of oligonucleotides, especially RNA, involved in protein synthesis. This is in contrast to conventional therapeutic methods, which seek to modulate protein activities by direct interaction between putative drugs and proteins.

In general, antisense methods involve determining the sequence of a coding oligonucleotide (e.g. mRNA) that encodes for a certain protein (sense strand), developing a relatively short oligomer that selectively binds to the sense strand, and introducing the oligomer into the intracellular environment. Antisense methods can predictably silence gene expression through a variety of mechanisms. In one such mechanism, the antisense strand blocks translation by competitively binding to the sense strand. In another mechanism, an antisense strand containing a stretch of DNA (e.g. phosphorothioate DNA) binds to the sense strand, whereby the DNA-RNA hybrid is recognized by RNAse H, an endonuclease that selectively cleaves the DNA-RNA hybrid, thereby reducing intracellular RNA levels. Another methodology involves the interaction between small double stranded RNA

oligomers and mRNA. In such mechanisms, interaction between the RISC complex, the antisense strand of the small double stranded RNA and intracellular mRNA results in cleavage and degradation of the mRNA.

As antisense molecules have become accepted as therapeutic and diagnostic agents, the need to produce oligonucleotides in large quantities has increased as well. The most commonly used antisense compounds to date have been oligonucleotides, phosphorothioate oligonucleotides and second generation oligonucleotides having one or more modified ribosyl sugar units, and more recently, ribosyl sugar units. The methods for making these three types of antisense oligomers are roughly similar, and include the phosphotriester method, as described by Reese, *Tetrahedron* 1978, *34*, 3143; the phosphoramidite method, as described by Beaucage, in *Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs*; Agrawal, ed.; Humana Press: Totowa, 1993, Vol. 20, 33-61; and the *H*-phosphonate method, as described by Froehler in *Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs* Agrawal, ed.; Humana Press: Totowa, 1993, Vol. 20, 63-80. Of these three methods, the phosphoramidite method has become a *de facto* standard in the industry.

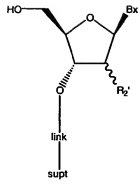
A typical oligonucleotide synthesis using phosphoramidite chemistry (i.e. the amidite methodology) is set forth below. First, a primer support is provided in a standard synthesizer column. The primer support is typically a solid support (supt) having a linker (link) covalently bonded thereto. It is common to purchase the primer support with a first 5'-protected nucleoside bonded thereto.



5'-blocked Primer-Support Bound Nucleoside

Primer support: bg is a 5'-blocking group, Bx is a nucleobase, R_2 is H, OH, OH protected with a removable protecting group, or a 2'-substituent, such as 2'-deoxy-2'-methoxyethoxy (2'-*O*-MOE), and link is the covalent linking group, which joins the nucleoside to the support, supt.

(A) The 5'-blocking group bg (e.g. 4,4'-dimethoxytrityl) is first removed (e.g. by exposing the 5'-blocked primer-support bound nucleoside to an acid), thereby producing a support-bound nucleoside of the formula:



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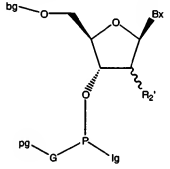
Activated primer support: wherein supt is the solid support, link is the linking group, Bx is a nucleobase, $R_{2'}$ is H, OH, OH protected with a removable protecting group, or a 2'-substituent.

(B) The column is then washed with acetonitrile, which acts to both "push" the regent (acid) onto the column, and to wash unreacted reagent and the removed 5'-blocking group (e.g. trityl alcohol) from the column.

(C) The primer support is then reacted with a phosphitylation reagent (amidite), which is dissolved in acetonitrile, the amidite having the formula:



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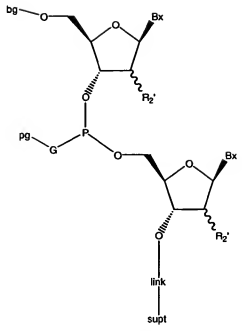
wherein bg is a 5'-blocking group, lg is a leaving group, G is O or S, pg is a phosphorus protecting group, and $R_{2'}$ and Bx have, independent of the analogous variables on the primer support, the same definitions as previously defined.

The product of this reaction is the support-bound phosphite dimer:



(Phosphite Dimer)

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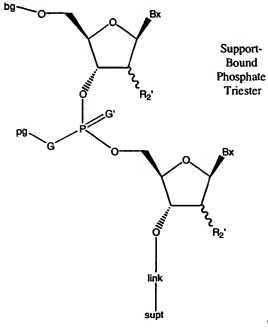
Support-bound wherein each of the variables bg, pg, G, $R_{2'}$ and Bx is independently defined above, link is the linker and supt is the support, as defined above.

(D) The support-bound dimer is then typically washed with acetonitrile.

(E) A capping reagent in acetonitrile is then added to the column, thereby capping unreacted nucleoside.

(F) The column is then washed again with acetonitrile.

(G) The support-bound dimer is then typically reacted with an oxidizing agent, such as a thiolating agent (e.g. phenylacetyl disulfide), in acetonitrile, to form a support-bound phosphate triester:



Support-Bound Phosphate Triester

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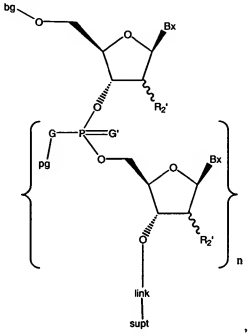
wherein G' is O or S and the other variables are defined herein.

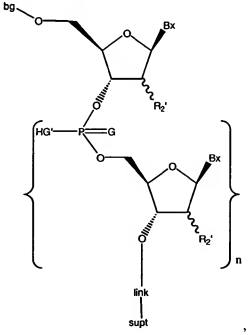
(H) The support-bound phosphate triester is then typically washed with acetonitrile.

Steps (A) – (F) are then repeated, if necessary, a sufficient number of times to prepare a support-bound, blocked oligonucleotide having the formula:

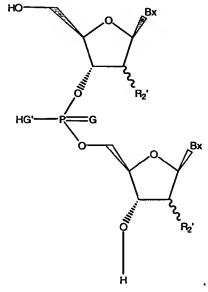


wherein n is a positive integer (typically about 7 to about 79).

The phosphorus protecting groups pg are then typically removed from the oligomer to produce a support-bound oligomer having the formula:



which, after washing with a suitable wash solvent, such as acetonitrile, is typically cleaved from the solid support, purified, 5'-deblocked, and further processed to produce an oligomer of the formula:



The person having skill in the art will recognize that G'H bound to a P(V) phosphorus is generally is ionized at physiologic pH, and that therefore, wherever G'H appears in the formulae above, or hereafter, G'' is synonymous therewith (the O' or S' being countered by a suitable cation, such as Na^+).

Typical methodologies for making oligonucleotides have not fundamentally changed since the development of the 5'-dimethoxytrityl group by Caruthers et al. (see patents, *infra*) and the cyanoethoxyphosphite by Köster et al. (patents, *infra*). While the coupling chemistry for oligonucleotide synthesis is relatively robust and reliable, it does suffer some drawbacks. For example, alternative chemistry using 5'-silyl protecting groups have been developed by Scaringe et al. for the preparation of RNA. However, this 5'-silyl protecting strategy is incompatible with the syntehsis of phosphorothioate oligonucleotides. For phosphoramidites with bulky 2'-substituents, such as methoxyethyl, the coupling efficiency to free 5'-OH residues on solid support is diminished. This change results form the steric hindrance in approach of the activated amidite to the support-bound 5'-OH, but also may result from incomplete removal of the 5'-DMT group in the previous cycle of synthesis. Additional evidence for this slow removal during the oligomerization process is the slow deprotection kinetics for 5'-DMT groups from sequences that end in T. Such sequences often take 4-10 times longer in contact with acid to remove the DMT group following HPLC purification.

Use of strong acid to remove the DMT introduces a class of impurity into the oligonucleotide. Strong acid treatment during synthesis induces hydrolysis of the protected purine bases from the sugar phosphate backbone, particularly deoxynucleotide residues. Treatment of the

HPLC-purified 5'-DMT oligonucleotide with strong acid also generates such apurinic sites. Given the first-order kinetics of DMT removal and the similarity in the pKa values of adenine, guanine and DMT groups, complete removal of DMT always generates some apurinic sites in the final product.

There is a need for an improved protecting group that can be removed by acids having higher pKa's than the acids required for removal of DMT.

There is also a need for an improved protecting group that can be removed under conditions that cause less depurination than those conditions required to remove DMT.

There is also a need for an improved protecting group that can act as a suitable hydrophobic handle during reverse phase high performance liquid chromatography.

There is also a need for an improved protecting group that can be manufactured by economically favorable processes.

There is also a need for a method of manufacturing a protecting group that can be removed under conditions that cause less depurination than the conditions required for removal of DMT.

There is also a need for a reagent capable of introducing a protecting group that can be removed under conditions that cause less depurination than those conditions required for removing DMT.

There is also a need for a reagent capable of introducing a protecting group that can be removed at higher pH than is required for removal of DMT.

There is also a need for a method of making a reagent capable of introducing a protecting group that can be removed using an acid having higher pKa than that required for removal of DMT.

SUMMARY OF THE INVENTION

The foregoing and further needs are met by embodiments of the present invention, which provide a reagent having the

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel process for manufacture of pixyl alcohol, pixylating reagents, pixyl-protected nucleosides, pixyl-protected nuceloside amidites (phosphitylating reagents), and pixyl-protected oligonucleotides. The present invention also provides methods for purification of oligonucleotides by reverse-phase liquid chromatography, wherein the oligonucleotides are pixylated with novel pixyl groups according to the present invention. The present invention also provides for removal of pixyl groups under conditions that are more mild than those required to remove the commonly-used trityl groups (DMT, MMT, etc.). The present invention this provides methods for oligonucleotide synthesis that are significantly better than those of the prior art, especially with respect to depurination. Additionally, certain 2'-protected ribonucleosides may be blocked at the 5'-position with novel pixyl groups according to the present invention. The 2'-protecting groups of such ribonucleosides may be selected from protecting groups that are orthogonal to the 5'-pixyl group, e.g.

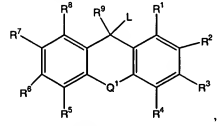
silyl protecting groups as discussed in more detail herein, or groups that are labile under conditions more acidic than those under which the pixyl derivatives are labile. Additionally, the present invention provides the advantage of providing an inexpensive and facile method for making pixyl groups, including derivatized pixyl groups having a wide range of substituent groups. These pixyl groups include thiopixyl groups, azapixyl groups (in which the O in the flourene ring system is replaced with NH, or alkylated or acylated nitrogen).

{insert description here}

The present invention is concerned with the general problem of protecting nucleosides during oligonucleotide synthesis.

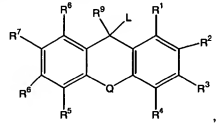
General Synthetic method

The present invention provides a new method for synthesis of pixylating reagents. In general, pixylating reagents according to the present invention have the formula:

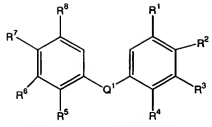


wherein R^1-R^8 are each, independently, H, alkyl, aryl, alkenyl, alkynyl, cyano, azido, halo, hydroxy, -C(=O)O-alkyl, -O-C(=O)-R^{10}, -C(=O)N(R^{10})R^{11}, -NHC(=O)R^{10}, -N(R^{10})R^{11}, -O-R^{12}, -S-R^{12}, or two or more groups R^1-R^8, together with the ring carbons to which they are bonded, combine to form a fused ring; R^9 is alkyl, alkenyl, alkynyl or aryl, wherein aryl may be substituted with one or more groups as defined by R^1; R^{10} is H or C_1-C_{24} alkyl, R^{11} is H or C_1-C_{24} alkyl, and R^{12} is C_1-C_{24} alkyl; and L is a leaving group; and Q is O, S, N(C=O)-R^{10}, or NR^{10}.

Thus, embodiments of the present invention provide a process of manufacturing a product compound of the formula:



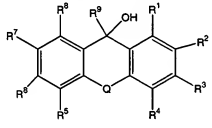
wherein the variables are defined above, said process comprising reacting a first compound of the formula:



with a second compound of the formula:

wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, $C(=O)OR^{13}$, or $C(=O)NR^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{24} alkyl; in the presence of a Friedel-Crafts acid;

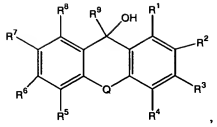
to produce an intermediate compound of the formula:



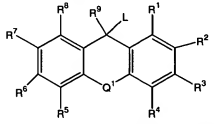
and reacting the intermediate compound with a reagent for introducing the leaving group L to form said product compound.

In some embodiments of the present invention, Q is O and R^2 and R^7 are other than H. In other embodiments of the invention, Q is O and R^2 and R^7 are alkyl, and R^1, R^3-R^6 and R^8 are each H. In some embodiments of the invention, R^9 is phenyl or optionally substituted phenyl. While not wishing to be bound by theory, it is believed that electron-releasing substituents, such as alkyl, are conductive to the present invention's being used as a protecting group capable of being removed at a pKa higher than that provided by the DMT group. Thus, in preferred embodiments of the invention, at least one of R^1-R^8 is an electron releasing group, such as methyl, ethyl, isopropyl, n-propyl, isobutyl, t-butyl, n-butyl, s-butyl, etc. In other preferred embodiments R^9 is a substituted phenyl in which the phenyl is substituted with an electron-releasing group such as C_1-C_{12} alkyl. In especially preferred embodiments, two of R^1-R^8 are alkyl, and R^9 is optionally substituted phenyl.

The present invention provides for novel pixyl derivatives of the formula:



, wherein the variable groups are as defined above. These derivatives may be converted to the corresponding pixylating agent:



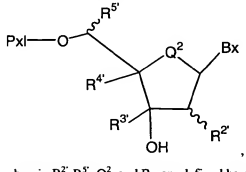
by reacting the alcohol with a suitable reagent for introducing L. Such a reagent includes: an acid chloride, such as acetyl chloride. The acid chloride may be dissolved in a solvent, such as toluene, acetonitrile or a chlorinated hydrocarbon such as methylene chloride.

Suitable Friedel-Crafts reagents include those known in the art, such as PX_3, wherein X is a halide. Specific Friedel-Crafts reagents include PCl_3 and PBr_3.

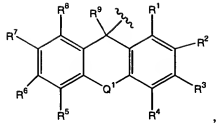
Suitable Friedel-Crafts acids include those known in the art, such as Lewis acids, including halides of metals, such as halides of zinc. A specific Friedel-Crafts acid suitable for the present invention is zinc chloride.

In particular embodiments of the invention, the novel pixyl groups are those in which Q is O, and at least one of R^1-R^8 is alkyl. In certain embodiments, novel pixyl groups are those in which Q is O, and at least two of R^1-R^8 are alkyl. In specific embodiments, novel pixyl groups are those in which Q is O and novel pixyl groups are those in which Q is O, and two of R^1-R^8 are alkyl, the others being H. In certain specific embodiments, Q is O, R^2 and R^7 are alkyl, such as methyl, ethyl, isopropyl, n-propyl, isobutyl, n-butyl, s-butyl, t-butyl, etc., and the remainder of the substituents are each H.

The present invention also includes methods of making nucleosides of the formula:



wherein $R^{2'}$-$R^{5'}$, Q^2, and Bx are defined herein. Pxl is the group:

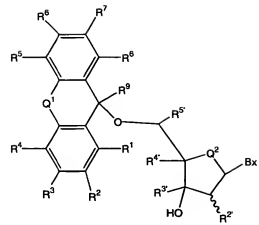


wherein the substituents R^1-R^9 are defined herein.

The electronic properties and pKa of the group can be modulated through substituetion of electron-donating or electron-withdrawing substituents on the phenyl or xanthyl (xanthenyl) rings. In some embodiments of the invention, the pKa of the 5'-pixyl group is matched with the pKa of the acid chosen to effect deprotection. In a best case, the pKa of the acid is higher than the pKa of the purine nucleobases. The inventors have found that the kinetics of removal for electron-donating dimethylpixyl groups is significantly faster than a dimethoxytrityl group. For example, the 5'-dimethylpixyl-2'-methoxyethylribothymidine has a half-life ($t_{1/2}$) of 101 minutes upon treatement with 5% methanol, whereas the corresponding 5'-dimethoxytrityl compound has a half-life of 420 minutes under the same conditions. This trend is expected to be followed by other di-alkylpixyl derivatives as described herein. The deprotection time for removal of the 5'-(2,7-dimethylpixyl)-MOE-T residue at the 5'-terminus of a synthetic oligonucleotide is <10 minutes, whereas the corresponding 5'-DMT group results in a deprotection time of about 40 minutes.

Pixyl groups may be used to synthesize oligonucleotides by methods analogous to those described hereinafter. In such cases, it is understood that one or more of the groups bg are replaced by the pixyl (Pxl) group as described herein. In some embodiments, only the terminal bg is Pxl, the remainder being DMT groups. The present inventors have found surprisingly that a terminal DMT group is especially difficult to remove when the nucleobase of the 5'-terminal nucleoside is thymidyl. Thus, in some embodiments of the present invention where the 5'-terminal nucleobase is thymidyl, bg for the 5'-terminus is Pxl, the other bg groups being some other blocking group, such as DMT. In certain embodiments of the present invention, the bg groups may be selected from among Pxl and other than Pxl, depending upon the nature of the nucleosides. In some cases, sequence-dependent effects may be observed. In such cases, the person having skill in the art will choose the most suitable protecting group, with the proviso that at least one will be Pxl, and in preferred embodiments that the last nucleoside to be added to the nascent chain will be a Pxl group.

The Pxl group also provides a convenient orthogonal protecting group for production of RNA. In certain embodiments, a protected nucleoside having the formula:



are provided (the substituents being defined herein) in which $R^{2'}$ is a protected OH. The OH protecting groups include those described by Scaringe et al. in US 6,008,400, US 6,111,086, and US 5,889,136 (each incorporated herein by reference) and by Reese in US 5,436,331 (incorporated herein by reference).

The pixyl group possesses hydrophobic character similar to that of the DMT group, such that an oligonucleotide having the 5'-*O* position protected with Pxl may be purified by reverse-phase liquid chromatography.

Acids for protecting group removal

The present invention provides the advantage of allowing removal of the 5'-protecting group under conditions that are more mild than those required to remove the DMT group. In particular, the present invention provides for removal of pixyl groups and their derivatives using acids that have higher pKa than dichloroacetic acid, and at rates significantly higher than those provided with the DMT/DCA combination. Specific acids that may be used to remove the pixyl group and its derivatives include: Acetic acid, Dibutylphosphoric acid, 2,2-Dichloropropionic acid, Dichloroacetic acid, Tetrazole, Salicylic acid, α-chlororbutyric acid, Butyric acid, Chloroacetic acid, Formic acid, Hexanoic acid, Heptanoic acid, Benzoic acid, Cyanoacetic acid, Pyruvic acid, Acetoacetic acid, Methoxyacetic acid, Levulinic acid, Methylthioacetic acid, Pivalic acid, Stearic acid, Oleic acid, Palmitic acid, Myristic acid, Malonic acid, Succinic acid, Adipic acid, Glutaric acid, Lactic acid, Citric acid, Malic acid, Pyruvic acid, α-chlororcaproic acid, and α-methylsuccinic acid.

In the context of the invention, the terms "oligomeric compound" and "oligomer" refer to a polymeric structure capable of hybridizing a region of a nucleic acid molecule (e.g. DNA, RNA or derivative). Each oligomer comprises a plurality of monomer subunits, and each monomer subunit in turn comprises a binding member and a backbone. The function of the binding member is to provide

sequence-specific binding to a target oligo- or polynucleotide. These interactions may be Watson-Crick hybridization, Hoogsteen base pairing, a combination of these mechanisms, or some other sequence-specific interaction.

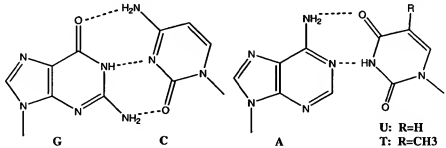
The function of the backbone is to hold the binding members in a spatial configuration amenable to sequence-specific binding. In general, the backbone comprises a skeletal member and a linking member. The skeletal member generally has separate sites to which the binding member and the linking member (linker) are bound. The linker joins skeletal members of adjacent monomer subunits, thereby establishing the sequence of the monomers.

In naturally occurring RNA, the binding member is a nucleosidic base, and the backbone comprises a sugar residue and a phosphate. The sugar residue, ribosyl, acts as the skeletal member, while the phosphate joins adjacent monomers through the 5'- and 3'- oxygen atoms on the ribosyl ring. The sugar is also bound to the nucleosidic base (base) at the 1'-position, the –β-D configuration predominating.

Naturally occurring DNA is analogous to RNA, except that the sugar is a 2'-deoxyribosyl.

Various combinations of binding member and backbone are known in the art. Together a binding member and a backbone form a monomeric subunit. Various types of monomeric subunits will be discussed in detail below.

As alluded above, one type of monomeric subunit known in the art is a nucleotide, which is a base-sugar-phosphate combination. The base portion of the nucleoside is normally a heterocyclic base moiety, also called a nucleobase. The two most common classes of such heterocyclic bases are purines and pyrimidines. The naturally occurring purine bases are guanine (G) and adenine (A), which are linked to the sugar through the 9-N nitrogen in the β-anomeric position on the sugar ring. The naturally occurring pyrimidine bases are uracil (U), thymine (T) and cytidine (C), which are linked to the sugar through the 1-N nitrogen. In DNA, Watson-Crick base pairing occurs between G and C, and between A and T, whereas in RNA, Watson-Crick base pairing occurs between G and C, and between A and U. The Watson-Crick base pairs for DNA and RNA are shown below.



In synthetic oligonucleotides, such as antisense therapeutics and diagnostics, one or more of the naturally occurring nucleobases may be replaced by an analogous binding member (nucleobase analog). Hereinafter, nucleobases and their analogs will be referred to collectively as bases. In

general, a nucleobase analog is a moiety that behaves like a nucleobase by providing sequence-specific binding to a target. Such binding generally occurs by hydrogen bonding between nucleobase ring constituents and/or exocyclic substituents, and may be analogous to Watson-Crick bonding, Hoogsteen bonding, some combination thereof, or some other regime.

In naturally occurring oligonucleotides, the sugar ring is β-D-ribosyl (RNA) or β-D-2'-deoxyribosyl (DNA). As alluded above, the hybridization behavior of DNA with RNA differs from the hybridization of RNA to RNA. This difference gives rise to different *in vitro* and *in vivo* effects. For example, DNA-RNA hybrids effectively bind to RNAse H, which results in scission of RNA. In contrast, RNA-RNA hybrids may be unwound by helicase, whereby the antisense strand is permitted to form a hybrid with mRNA. The exogenous RNA-mRNA hybrid interacts with one or more members of the RISC complex, which effects mRNA scission.

Synthetic sugars and sugar analogs are designed to adopt certain spatial conformations that resemble DNA, RNA or some structure intermediate between these conformations. Again, the sugar or sugar analog functions as a sort of platform to hold the base in the correct orientation to interact with bases on the opposite strand. The sugar or sugar analog (collectively skeletal members) also provides binding sites for the linking groups, which join the monomeric units together to form the oligomer. The conformation of the sugar or sugar analog greatly influences the spatial orientations of the bases and linking groups, and also greatly influences the shape of the antisense-sense hybrid in solution. This conformational influence can have an important impact on the efficacy of the antisense compound in modulation of gene expression.

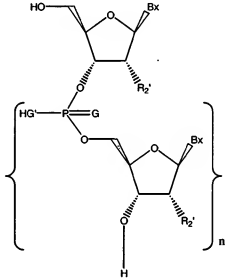
Naturally occurring nucleosides are linked to one another via a phosphoryl diester linker. Antisense compounds may be prepared using phosphoryl diester linkers, which are generally suitable for diagnostic and other nuclease-free uses. However, antisense therapeutic compounds advantageously comprise at least one phosphorothioate linker, owing to the latter's superior nuclease stability. Both phosphoryl and phosphorothioate linkers are generally referred to as phosphate diester linkers. When a plurality of nucleosides are linked by successive phosphate diester linkers, the resulting oligomer is called an oligonucleotide.

Synthetic linking groups have been developed, some of which employ phosphorus in the linking portion, and some of which do not. For example, PNAs (peptide nucleic acids) use the uncharged amide bond instead of a phosphate diester bond to link adjacent sugar moieties. In the case of PNAs, the linker may perform the linking function as well as the functions performed by the sugar moiety in naturally occurring oligonucleotides. Other examples of linking groups exist, as described in more detail herein.

As alluded above, synthetic oligonucleotides may be modified extensively from their natural form for use in antisense therapeutics. The most commonly occurring modifications include the phosphorothioate backbone, the presence of various substituents on the sugar moiety, and modification of bases. Many of these variations will be discussed below.

In the broadest sense, the term "oligonucleotide" refers to an oligomer having a plurality of skeletal members, e.g. sugar units (ribosyl, deoxyribosyl, arabinosyl, modified sugar unit, etc.) linked by phosphate diester linkers (i.e. phosphoryl or thiophosphoryl diester), and having bases for establishing binding to complementary oligomer strands. In some embodiments of the invention, an oligonucleotide may contain both phosphoryl diester and phosphorothioate linkers. In other embodiments, the linkers are all phosphorothioate linkers. While phosphoryl linkers are the naturally occurring type of linkers in oligonucleotides, thiophosphate linkers are known to confer nuclease stability to oligonucleotides cells. Hence, it is often preferred to prepare oligonucleotides with at least a portion of the phosphate diester moieties replaced by phosphorothioate diester moieties.

Oligonucleotides according to embodiments of the present invention are represented by formula 1:

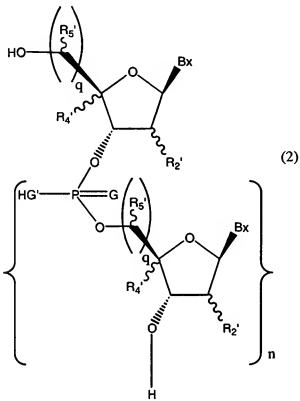


, or tautomers, salts or solvates thereof. In formula 1, each G and G' is independently O or S, each $R_{2'}$ is independently H or OH, n is an integer and each Bx is independently a nucleobase as described in greater detail herein. Thus the repeating backbone unit is a ribosyl ring linked to a phosphate or phosphorothioate linker. Selectivity for a particular target sequence is achieved by modification of the sequence of Bx units. This procedure is discussed in greater detail herein.

The 2'-position may be H (i.e. 2'-deoxyribosyl) or OH (ribosyl). While it is possible for all $R_{2'}$ units to be OH, e.g. where the oligomers will be used in siRNA applications, it is often desirable for all or part of the oligomer to be 2'-deoxy. In preferred embodiments of the present invention, each of the $R_{2'}$ groups is H. In other cases, a contiguous stretch sugars are 2'-deoxy, while one or more stretches of the remainder of the oligonucleotide contain ribosyl or 2'-modified ribosyl sugars, as described in more detail herein. It has been found that oligonucleotides containing a stretch of deoxy ribosyl nucleotides are able to recruit RNase H, as described in greater detail herein.

Formula 1 depicts the simplest oligonucleotides, which are also referred to in the art as "first generation" oligonucleotides. Other oligonucleotides are possible, and are encompassed within the

meaning of "oligonucleotide" as used herein. In particular, oligonucleotides may contain repeating units where the standard ribosyl unit is replaced with a substituted ribosyl unit (e.g. a 2'-deoxy-2'-substituted ribosyl unit), where the ribosyl unit is replaced by a different sugar entirely (e.g. an arabinosyl or erythrosyl unit), or where the ribosyl unit is replaced by a bridged sugar unit. A general formula for an oligonucleotide of this type is depicted in Formula 2.



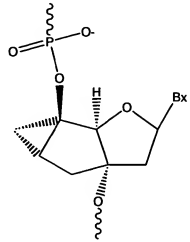
(2)

, or tautomers, salts or solvates thereof. In formula 2, G, G', Bx and n have the same meanings as in formula 1. The squiggly lines (~~~) ring indicate that the corresponding substituents may be in either the down or up configuration. The value of q may be 0 or 1. $R_{2'}$ may be H, OH, a reversibly protected OH, a 2'-substituent, or may form, together with $R_{4'}$, a bridge. $R_{4'}$ is either H or, together with $R_{2'}$ or $R_{5'}$, forms a bridge.

The person skilled in the art will recognize that when $R_{2'}$ is in the down configuration and q' is 1, the ring is a ribosyl ring, whereas when $R_{2'}$ is in the up configuration and q' is 1, the ring is an arabinosyl ring. Likewise, when q' is 0 and R_2' is in the down configuration, the ring is an erythrosyl ring. When R_2' and R'$_4$ are joined to form a bridge, the ring is called a locked nucleic acid (LNA), as described in greater detail herein. In some embodiments, the bridge formed by R_2' and R'$_4$ is R_2'-O-$(CH_2)_r$-R'$_4$ (wherein r is 1 or 2) or R_2'-CH_2-O-CH_2-R'$_4$ (the use of R_2' and R'$_4$ in the sub-formulae indicating the points of attachment.) LNA may be present in either α-L- or β-D- conformation. See Vester et al., "LNAzymes: Incorporation of LNA-Type Monomers into DNAzymes Markedly Increases RNA Cleavage," Journal of the American Chemical Society, 2002, 124, 13682-3. Each of these analogs possesses a number of useful characteristics, including resistance to exonuclease

activity, induction if endonuclease activity (e.g. by RNAse H, the RISC complex, etc.) and modulation of hybridization.

When R_4 and R_5 form a bridge, they may form, along with the sugar ring to which they are attached, a tricyclic ring. Tricyclic nucleosides of the structure:



are described by Rennenberg et al. in Nucleic Acids Research, 30(13), 2751-7 (2002). One skilled in the art will recognize that the analogous phosphorothioates, and 2'-substituted tricyclic deoxynucleosides may be prepared by methods analogous to those taught by Rennenberg et al., as modified by the teaching herein. In particular, the phosphorothioates may be prepared by substituting a sulfurizing oxidant (a.k.a. a sulfur transfer reagent, such a phenyl acetyl disulfide) for the oxidizing agent taught by Rennenberg et al. The 2'-substituted tricyclic deoxynucleosides may be prepared from the analogous 2'-substituted deoxynucleosides, using a 2'-OH protecting group in the case of ribonucleic acid.

The variable Sug, as used herein, refers to a sugar ring or a modified sugar ring. Sugar rings include ribosyl, 2'-deoxyribosyl, arabinosyl, erythrosyl and other modified sugar rings, such as bicyclic and tricyclic ring systems. Modified sugar rings include the foregoing sugar rings as modified per the description herein, e.g. at the 2'-position, or by a bridge between the 2'- and 4'-positions as described in further detail herein.

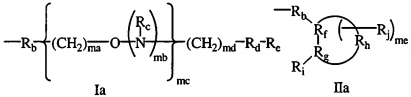
Certain oligonucleotides that utilized arabino-pentofuranosyl nucleotides as building blocks have been described. Damha et. al., J.A.C.S., 1998, 120, 12976-12977; and Damha et. al., Bioconjugate Chem., 1999, 10, 299-305.

Suitable 2'-substituents corresponding to R_2 include: F, O-alkyl (e.g. O-methyl), S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl; O-alkynyl, S-alkynyl, N-alkynyl; O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl or alkynyl, respectively. Particularly preferred are $O[(CH_2)_gO]_hCH_3$, $O(CH_2)_gOCH_3$, $O(CH_2)_gNH_2$, $O(CH_2)_gCH_3$, $O(CH_2)_gONH_2$, and $O(CH_2)_gON[(CH_2)_gCH_3)]_2$, where g and h are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower

alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred 2'-modification is 2'-deoxy-2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-*O*-(2-methoxyethyl) or 2'-MOE ribosyl) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, *78*, 486-504). Other preferred modifications include 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_3)_2$.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide.

Further representative substituent groups include groups of formula I_a or II_a:



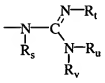
Ia IIa

wherein:

R_b is O, S or NH;

R_d is a single bond, O or C(=O);

R_e is C_1-C_{10} alkyl, $N(R_k)(R_m)$, $N(R_k)(R_n)$, $N=C(R_p)(R_q)$, $N=C(R_p)(R_r)$ or has formula III_a;



IIIa

each R_s, R_t, R_u and R_v is, independently, hydrogen, $C(O)R_w$, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein

the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_u and R_v, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each R_w is, independently, substituted or unsubstituted C_1-C_{10} alkyl, trifluoromethyl, cyanoethyloxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_k is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_p is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+, $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or R_m and R_n, together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

R_t is OR_z, SR_z, or $N(R_z)_2$;

each R_z is, independently, H, C_1-C_8 alkyl, C_1-C_8 haloalkyl, C(=NH)N(H)R_u, C(=O)N(H)R_u or OC(=O)N(H)R_u;

R_f, R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$ OR_k, halo, SR_k or CN;

m_a is 1 to about 10;

each mb is, independently, 0 or 1;

mc is 0 or an integer from 1 to 10;

md is an integer from 1 to 10;

me is from 0, 1 or 2; and

provided that when mc is 0, md is greater than 1.

Representative substituents groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy

Oligonucleotides," hereby incorporated by reference in its entirety. Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Modified Oligonucleotides that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include $O[(CH_2)_gO]_hCH_3$, $O(CH_2)_gOCH_3$, $O(CH_2)_gNH_2$, $O(CH_2)_gCH_3$, $O(CH_2)_gONH_2$ and $O(CH_2)_gON[(CH_2)_gCH_3)]_2$ where g and h are from 1 to about 10.

Some preferred oligomeric compounds of the invention contain at least one nucleoside having one of the following substituent groups: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligomeric compound, or a group for improving the pharmacodynamic properties of an oligomeric compound, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE] (Martin et al., Helv. Chim. Acta, 1995, 78, 486), i.e., an alkoxyalkoxy group. A further preferred modification is 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE. Representative aminooxy substituent groups are described in co-owned United States Patent Application serial number 09/344,260, filed June 25, 1999, entitled "Aminooxy-Functionalized Oligomers"; and United States Patent Application serial number 09/370,541, filed August 9, 1999, entitled "Aminooxy-Functionalized Oligomers and Methods for Making Same;" hereby incorporated by reference in their entirety.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on nucleosides and oligomers, particularly the 3' position of the sugar on the 3' terminal nucleoside or at a 3'-position of a nucleoside that has a linkage from the 2'-position such as a 2'-5' linked oligomer and at the 5' position of a 5' terminal nucleoside. Oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053I 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/468,037, filed on June 5, 1995, also herein incorporated by reference.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent 6,147,200 which is hereby incorporated by reference in its entirety. Representative dimethylaminoethyloxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethyloxyethyl-Modified Oligonucleotides", filed August 6, 1999, hereby incorporated by reference in its entirety. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. The respective ends of this linear polymeric structure can be joined to form a circular structure by hybridization or by formation of a covalent bond, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the oligonucleotide. The normal internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage.

While the present invention may be adapted to produce oligonucleotides for any desired end use (e.g. as probes for us in the polymerase chain reaction), one preferred use of the oligonucleotides is in antisense therapeutics. One mode of action that is often employed in antisense therapeutics is the so-called RNAse H mechanism, whereby a strand of DNA is introduced into a cell, where the DNA hybridizes to a strand of RNA. The DNA-RNA hybrid is recognized by an endonuclease, RNAse H, which cleaves the RNA strand. In normal cases, the RNA strand is messenger RNA (mRNA), which, after it has been cleaved, cannot be translated into the corresponding peptide or protein sequence in the ribosomes. In this way, DNA may be employed as an agent for modulating the expression of certain genes.

It has been found that by incorporating short stretches of DNA into an oligonucleotide, the RNAse H mechanism can be effectively used to modulate expression of target peptides or proteins. In some embodiments of the invention, an oligonucleotide incorporating a stretch of DNA and a stretch of RNA or 2'-modified RNA can be used to effectively modulate gene expression. In preferred embodiments, the oligonucleotide comprises a stretch of DNA flanked by two stretches of 2'-modified RNA. Preferred 2'-modifications include 2'-*O*-methyl and 2'-*O*-methoxyethyl as described herein.

The ribosyl sugar moiety has also been extensively studied to evaluate the effect its modification has on the properties of oligonucleotides relative to unmodified oligonucleotides. The 2'-position of the sugar moiety is one of the most studied sites for modification. Certain 2'-substituent groups have been shown to increase the lipohpilicity and enhance properties such as binding affinity to target RNA, chemical stability and nuclease resistance of oligonucleotides. Many of the modifications at the 2'-position that show enhanced binding affinity also force the sugar ring into the C_3-endo conformation.

RNA exists in what has been termed "A Form" geometry while DNA exists in "B Form" geometry. In general, RNA:RNA duplexes are more stable, or have higher melting temperatures (Tm)

than DNA:DNA duplexes (Sanger *et al., Principles of Nucleic Acid Structure,* **1984**, Springer-Verlag; New York, NY.; Lesnik *et al., Biochemistry,* **1995**, *34*, 10807-10815; Conte *et al., Nucleic Acids Res.*, **1997**, *25*, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle *et al., Nucleic Acids Res.*, **1993**, *21*, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' *endo* pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. On the other hand, deoxy nucleic acids prefer a C2' *endo* sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli *et al., Biochemistry*, **1996**, *35*, 8489-8494).

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle *et al., Nucleic Acids Res.*, **1993**, *21*, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane *et al., Eur. J. Biochem.*, **1993**, *215*, 297-306; Fedoroff *et al., J. Mol. Biol.*, **1993**, *233*, 509-523; Gonzalez *et al., Biochemistry*, **1995**, *34*, 4969-4982; Horton *et al., J. Mol. Biol.*, **1996**, *264*, 521-533). The stability of a DNA:RNA hybrid is central to antisense therapies as the mechanism requires the binding of a modified DNA strand to a mRNA strand. To effectively inhibit the mRNA, the antisense DNA should have a very high binding affinity with the mRNA. Otherwise the desired interaction between the DNA and target mRNA strand will occur infrequently, thereby decreasing the efficacy of the antisense oligonucleotide.

Various synthetic modifications have been proposed to increase nuclease resistance, or to enhance the affinity of the antisense strand for its target mRNA (Crooke *et al., Med. Res. Rev.*, **1996**, *16*, 319-344; De Mesmaeker *et al., Acc. Chem. Res.*, **1995**, *28*, 366-374). A variety of modified phosphorus-containing linkages have been studied as replacements for the natural, readily cleaved phosphodiester linkage in oligonucleotides. In general, most of them, such as the phosphorothioate, phosphoramidates, phosphonates and phosphorodithioates all result in oligonucleotides with reduced binding to complementary targets and decreased hybrid stability.

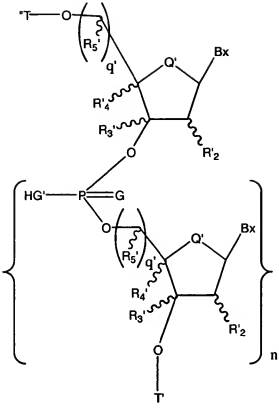
One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2'-methoxyethoxy (MOE, 2'-$OCH_2CH_2OCH_3$) side chain (Baker *et al., J. Biol. Chem.*, **1997**, *272*, 11944-12000; Freier *et al., Nucleic Acids Res.*, **1997**, *25*, 4429-4443). One of the immediate advantages of the MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as *O*-methyl, *O*-propyl, and *O*-aminopropyl (Freier and Altmann, *Nucleic Acids Research,* (1997) 25:4429-4443). 2'-*O*-methoxyethyl-substituted oligonucleotides also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, **1995,** *78*, 486-504;

Altmann *et al., Chimia*, **1996**, *50*, 168-176; Altmann *et al., Biochem. Soc. Trans.*, **1996**, *24*, 630-637; and Altmann *et al., Nucleosides Nucleotides*, **1997**, *16*, 917-926). Relative to DNA, they display improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides with 2'-*O*-methoxyethyl-ribonucleoside wings and a central DNA-phosphorothioate window also have been shown to effectively reduce the growth of tumors in animal models at low doses. MOE substituted oligonucleotides have shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

LNAs (oligonucleotides wherein the 2' and 4' positions are connected by a bridge) also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the **3'-endo** conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands. LNAs may be in either the α-L- or the β-D- conformation. Vester et al., J.A.C.S, 124 (2002) 13682-13683.

LNAs in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably an alkylene ($(-CH_2-)_n$) group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10 C), stability towards 3'-exonucleolytic degradation and good solubility properties. Other preferred bridge groups include the 2'-CH_2OCH_2-4' bridge.

While the present invention is concerned primarily with oligonucleotides, some oligonucleotide mimetics may, with appropriate changes to the starting materials, also be prepared by processes according to the present invention. Oligonucleotide mimetics include compounds in which the oligonucleotide sugar has been replaced with a heterocyclic or carbocyclic ring structure. Such compounds are depicted in Formula 3, below.



(3)

, and tautomers, salts and solvates thereof.

In Formula 3, G, G', Bx, n, R_2' and R_4' each have the meanings previously defined. In addition, R_5' may form, together with R_4', a ring structure, which optionally includes another ring and R_3' is H or a substituent group. The groups T' and T" are each H, or conjugate groups, such as protecting groups and substituents. Each Q' is independently O, S, NR'", $C(R'")_2$, or $-CR'"=CR'"-$, where each R'" is H, alkyl, or where two R'" groups are on the same or adjacent carbon atoms, they may form a carbocyclic or heterocyclic ring, wherein the ring contains one or two of N, O or S. Preferred values of R'" are H and C_1-C_4 alkyl.

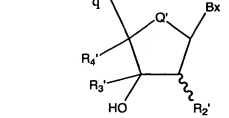
The foregoing oligonucleotides and oligonucleotide mimetics may be manufactured by any art-recognized method of forming phosphate diester or phosphorothioate diester linkages between successive nucleoside or nucleoside mimetic units. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

A preferred process of synthesizing oligomeric compounds utilizes phosphoramidite chemistry on a support media. The phosphoramidites can modified at the heterocyclic base, the sugar, or both positions to enable the synthesis of oligonucleotides and modified oligonucleotides.

Illustrative examples of the synthesis of particular modified oligonucleotides may be found in the following U.S. patents or pending patent applications, each of which is commonly assigned with this application: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Patent No. 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Patents Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Patent No. 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Patent No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Patent No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Patent No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Patent No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No. 5,554,746, drawn to oligonucleotides having β-lactam backbones; U.S. Patent No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Patent No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Patents Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Patent No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Patent No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Patents Nos. 5,223,168, issued June 29, 1993, and 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Patent Nos. 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and U.S. patent application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to, inter alia, methods of synthesizing 2'-fluoro-oligonucleotides.

The phosphoramidite method is as follows:

Phosphoramidites are prepared by reacting a suitable nucleoside or modified nucleoside (formula 4) with a phosphorodiamidite (formula 5) to form a phosphoramidite (formula 6).



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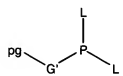

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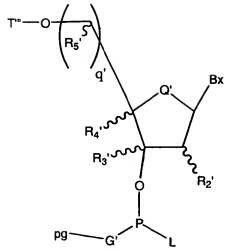
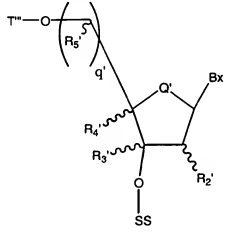


(6)

Each of the variables Q', Bx, $R_{2'}$, $R_{3'}$, $R_{4'}$, $R_{5'}$, G', and q' is as previously defined. L is an amine leaving group; pg is a phosphorus protecting group; and T'" is a hydroxyl protecting group, each as more specifically defined herein.

A support-bound nucleoside of Formula 7 is first deprotected at the 5'-position (resulting in a free 5'-OH group), after which a first amidite is coupled to a support-bound nucleoside to form a support-bound dimer of Formula 8, which is then oxidized, and subjected to a capping step to form a support bound dimer of Formula 9.



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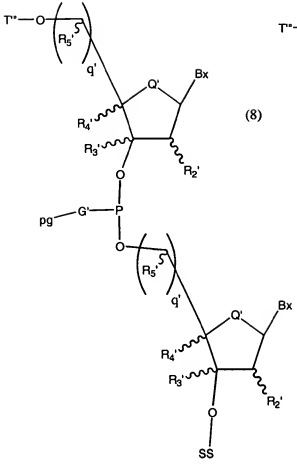

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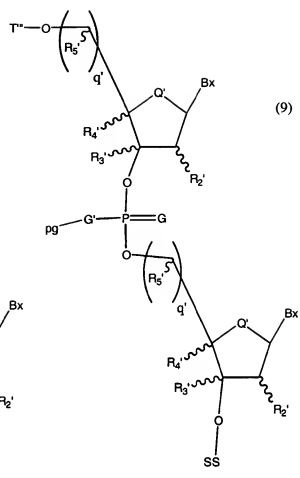
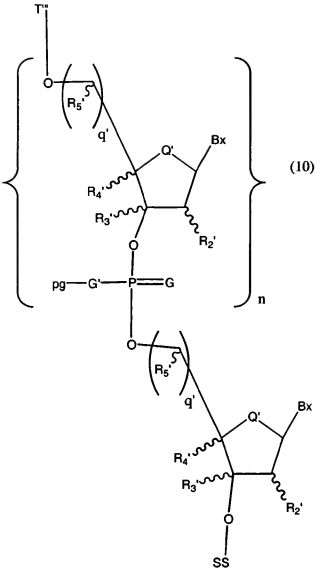


(9)

The 5'-deprotection, coupling, oxidation and capping steps are then repeated n-2 times to form a support-bound oligomer of Formula 10.



This compound is then cleaved from the solid support, 5'-deprotected, and purified to yield an oligomer of Formula (3), wherein T' is H. The oligonucleotide may then be further derivatized, purified, precipitated, or otherwise treated, as described in more detail herein.

In each of the foregoing Formulae, SS represents a support bound to the 3'-terminal nucleoside by a cleavable linker, each pg is a phosphorus protecting group as defined herein, n is an integer, G and G' are independently O or S, and each Bx, $R_{2'}$, $R_{3'}$, R'_4, $R_{5'}$, Q', and q' is independently as defined in Formula 3.

In addition to phosphate diester and phosphorothioate diester linkages, other linkers are known in the art. While the primary concern of the present invention has to do with phosphate diester and phosphorothioate diester oligonucleotides, chimeric compounds having more than one type of linkage, as well as oligomers having non-phosphate/phosphorothioate diester linkages as described in

further detail below, are also contemplated in whole or in part within the context of the present invention.

Exemplary non-phosphate/phosphorothioate diester linkages contemplated within the skill of the art include: phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates. Additional linkages include: thiodiester (-O-C(O)-S-), thionocarbamate (-O-C(O)(NJ)-S-), siloxane (-O-Si(J)$_2$-O-), carbamate (-O-C(O)-NH- and -NH-C(O)-O-), sulfamate (-O-S(O)(O)-N- and -N-S(O)(O)-N-, morpholino sulfamide (-O-S(O)(N(morpholino)-), sulfonamide (-O-SO_2-NH-), sulfide (-CH_2-S-CH_2-), sulfonate (-O-SO_2-CH_2-), N,N'-dimethylhydrazine (-CH_2-N(CH_3)-N(CH_3)-), thioformacetal (-S-CH_2-O-), formacetal (-O-CH_2-O-), thioketal (-S-C(J)$_2$-O-), ketal (-O-C(J)$_2$-O-), amine (-NH-CH_2-CH_2-), hydroxylamine (-CH_2-N(J)-O-), hydroxylimine (-CH=N-O-), and hydrazinyl (-CH_2-N(H)-N(H)-).

In each of the foregoing substructures relating to internucleoside linkages, J denotes a substituent group which is commonly hydrogen or an alkyl group or a more complicated group that varies from one type of linkage to another.

In addition to linking groups as described above that involve the modification or substitution of the -O-P-O- atoms of a naturally occurring linkage, included within the scope of the present invention are linking groups that include modification of the 5'-methylene group as well as one or more of the -O-P-O- atoms. Linkages of this type are well documented in the prior art and include without limitation the following: amides (-CH_2-CH_2-N(H)-C(O)) and -CH_2-O-N=CH-;and alkylphosphorus (-C(J)$_2$-P(=O)(OJ)-C(J)$_2$-C(J)$_2$-). J is as described above.

Synthetic schemes for the synthesis of the substitute internucleoside linkages described above are disclosed in: U.S. Patent Nos. 5,466,677; 5,034,506; 5,124,047; 5,278,302; 5,321,131; 5,519,126; 4,469,863; 5,455,233; 5,214,134; 5,470,967; 5,434,257. Additional background information relating to internucleoside linkages can be found in: WO 91/08213; WO 90/15065; WO 91/15500; WO 92/20822; WO 92/20823; WO 91/15500; WO 89/12060; EP 216860; PCT/US 92/04294; PCT/US 90/03138; PCT/US 91/06855; PCT/US 92/03385; PCT/US 91/03680; U.S. Application Nos. 07/990,848; 07,892,902; 07/806,710; 07/763,130; 07/690,786; Stirchak, E.P., et al., Nucleic Acid Res., 1989, 17, 6129-6141; Hewitt, J.M., et al., 1992, 11, 1661-1666; Sood, A., et al., J. Am. Chem. Soc., 1990, 112, 9000-9001; Vaseur, J.J. et al., J. Amer. Chem. Soc., 1992, 114, 4006-4007; Musichi, B., et al., J. Org. Chem., 1990, 55, 4231-4233; Reynolds, R.C., et al., J. Org. Chem., 1992, 57, 2983-2985; Mertes, M.P., et al., J. Med. Chem., 1969, 12, 154-157; Mungall, W.S., et al., J. Org. Chem., 1977, 42, 703-706; Stirchak, E.P., et al., J. Org. Chem., 1987, 52, 4202-4206; Coull, J.M., et al., Tet. Lett., 1987, 28, 745; and Wang, H., et al., Tet. Lett., 1991, 32, 7385-7388.

Phosphoramidites used in the synthesis of oligonucleotides are available from a variety of commercial sources (included are: Glen Research, Sterling, Virginia; Amersham Pharmacia Biotech Inc., Piscataway, New Jersey; Cruachem Inc., Aston, Pennsylvania; Chemgenes Corporation, Waltham, Massachusetts; Proligo LLC, Boulder, Colorado; PE Biosystems, Foster City California; Beckman Coulter Inc., Fullerton, California). These commercial sources sell high purity phosphoramidites generally having a purity of better than 98%. Those not offering an across the board purity for all amidites sold will in most cases include an assay with each lot purchased giving at least the purity of the particular phosphoramidite purchased. Commercially available phosphoramidites are prepared for the most part for automated DNA synthesis and as such are prepared for immediate use for synthesizing desired sequences of oligonucleotides. Phosphoramidites may be prepared by methods disclosed by e.g. Caruthers et al. (US 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418) and Köster et al. (US RE 34,069).

Oligonucleotides are generally prepared, as described above, on a support medium, e.g. a solid support medium. In general a first synthon (e.g. a monomer, such as a nucleoside) is first attached to a support medium, and the oligonucleotide is then synthesized by sequentially coupling monomers to the support-bound synthon. This iterative elongation eventually results in a final oligomeric compound or other polymer such as a polypeptide. Suitable support media can be soluble or insoluble, or may possess variable solubility in different solvents to allow the growing support bound polymer to be either in or out of solution as desired. Traditional support media such as solid supports are for the most part insoluble and are routinely placed in reaction vessels while reagents and solvents react with and/or wash the growing chain until the oligomer has reached the target length, after which it is cleaved from the support and, if necessary further worked up to produce the final polymeric compound. More recent approaches have introduced soluble supports including soluble polymer supports to allow precipitating and dissolving the iteratively synthesized product at desired points in the synthesis (Gravert et al., Chem. Rev., 1997, 97, 489-510).

The term support media (support) is intended to include all forms of support known to the art skilled for the synthesis of oligomeric compounds and related compounds such as peptides. Some representative support media that are amenable to the methods of the present invention include but are not limited to the following: controlled pore glass (CPG); oxalyl-controlled pore glass (see, e.g., Alul, et al., Nucleic Acids Research 1991, 19, 1527); silica-containing particles, such as porous glass beads and silica gel such as that formed by the reaction of trichloro-[3-(4-chloromethyl)phenyl]propylsilane and porous glass beads (see Parr and Grohmann, *Angew. Chem. Internal. Ed.* 1972, **11**, 314, sold under the trademark "PORASIL E" by Waters Associates, Framingham, Mass., USA); the mono ester of 1,4-dihydroxymethylbenzene and silica (see Bayer and Jung, *Tetrahedron Lett.*, 1970, 4503, sold under the trademark "BIOPAK" by Waters Associates); TENTAGEL (see, e.g., Wright, et al., Tetrahedron Letters 1993, 34, 3373); cross-linked styrene/divinylbenzene copolymer beaded matrix or POROS, a copolymer of polystyrene/divinylbenzene (available from Perceptive Biosystems); soluble

support media, polyethylene glycol PEG's (see Bonora et al., Organic Process Research & Development, 2000, 4, 225-231).

Further support media amenable to the present invention include without limitation PEPS support a polyethylene (PE) film with pendant long-chain polystyrene (PS) grafts (molecular weight on the order of 10^6, (see Berg, *et al., J. Am. Chem. Soc.*, 1989, 111, 8024 and International Patent Application WO 90/02749),). The loading capacity of the film is as high as that of a beaded matrix with the additional flexibility to accommodate multiple syntheses simultaneously. The PEPS film may be fashioned in the form of discrete, labeled sheets, each serving as an individual compartment. During all the identical steps of the synthetic cycles, the sheets are kept together in a single reaction vessel to permit concurrent preparation of a multitude of peptides at a rate close to that of a single peptide by conventional methods. Also, experiments with other geometries of the PEPS polymer such as, for example, non-woven felt, knitted net, sticks or microwell plates have not indicated any limitations of the synthetic efficacy.

Further support media amenable to the present invention include without limitation particles based upon copolymers of dimethylacrylamide cross-linked with N,N'-bisacryloylethylenediamine, including a known amount of *N*-tertbutoxycarbonyl-beta-alanyl-*N*'-acryloylhexamethylenediamine. Several spacer molecules are typically added via the beta alanyl group, followed thereafter by the amino acid residue subunits. Also, the beta alanyl-containing monomer can be replaced with an acryloyl safcosine monomer during polymerization to form resin beads. The polymerization is followed by reaction of the beads with ethylenediamine to form resin particles that contain primary amines as the covalently linked functionality. The polyacrylamide-based supports are relatively more hydrophilic than are the polystyrene-based supports and are usually used with polar aprotic solvents including dimethylformamide, dimethylacetamide, N-methylpyrrolidone and the like (see Atherton, *et al., J. Am. Chem. Soc.*, 1975, **97**, 6584, *Bioorg. Chem.* 1979, **8**, 351, and J. C. S. Perkin I 538 (1981)).

Further support media amenable to the present invention include without limitation a composite of a resin and another material that is also substantially inert to the organic synthesis reaction conditions employed. One exemplary composite (see Scott, *et al., J. Chrom. Sci.*, 1971, **9**, 577) utilizes glass particles coated with a hydrophobic, cross-linked styrene polymer containing reactive chloromethyl groups, and is supplied by Northgate Laboratories, Inc., of Hamden, Conn., USA. Another exemplary composite contains a core of fluorinated ethylene polymer onto which has been grafted polystyrene (see Kent and Merrifield, *Israel J. Chem.* 1978, **17**, 243 and van Rietschoten in *Peptides 1974*, Y. Wolman, Ed., Wiley and Sons, New York, 1975, pp. 113-116). Contiguous solid supports other than PEPS, such as cotton sheets (Lebl and Eichler, *Peptide Res.* 1989, **2**, 232) and hydroxypropylacrylate-coated polypropylene membranes (Daniels, *et al., Tetrahedron Lett.* 1989, 4345). Acrylic acid-grafted polyethylene-rods and 96-microtiter wells to immobilize the growing peptide chains and to perform the compartmentalized synthesis. (Geysen, *et al., Proc. Natl. Acad. Sci. USA*, 1984, **81**, 3998). A "tea bag" containing traditionally-used polymer beads. (Houghten, *Proc.*

Natl. Acad. Sci. USA, 1985, **82**, 5131). Simultaneous use of two different supports with different densities (Tregear, *Chemistry and Biology of Peptides*, J. Meienhofer, ed., Ann Arbor Sci. Publ., Ann Arbor, 1972 pp. 175-178). Combining of reaction vessels via a manifold (Gorman, *Anal. Biochem.*, 1984, **136**, 397). Multicolumn solid-phase synthesis (*e.g.*, Krchnak, *et al.*, *Int. J. Peptide Protein Res.*, 1989, **33**, 209), and Holm and Meldal, in "Proceedings of the 20th European Peptide Symposium", G. Jung and E. Bayer, eds., Walter de Gruyter & Co., Berlin, 1989 pp. 208-210). Cellulose paper (Eichler, *et al.*, *Collect. Czech. Chem. Commun.*, 1989, 54, 1746). Support mediated synthesis of peptides have also been reported (see, *Synthetic Peptides: A User's Guide*, Gregory A. Grant, Ed. Oxford University Press 1992; US-A-4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; 5,132,418; 4,725,677 and Re-34,069.)

Support bound oligonucleotide synthesis relies on sequential addition of nucleotides to one end of a growing chain. Typically, a first nucleoside (having protecting groups on any exocyclic amine functionalities present) is attached to an appropriate glass bead support and activated phosphite compounds (typically nucleotide phosphoramidites, also bearing appropriate protecting groups) are added stepwise to elongate the growing oligonucleotide. Additional methods for solid-phase synthesis may be found in Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Köster U.S. Patents Nos. 4,725,677 and Re. 34,069.

Commercially available equipment routinely used for the support media based synthesis of oligomeric compounds and related compounds is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. Suitable solid phase techniques, including automated synthesis techniques, are described in F. Eckstein (ed.), Oligonucleotides and Analogues, a Practical Approach, Oxford University Press, New York (1991).

In general, the phosphorus protecting group (pg) is an alkyl group or a β-eliminable group having the formula $-CH_2CH_2-G_w$, wherein G_w is an electron-withdrawing group. Suitable examples of pg that are amenable to use in connection with the present invention include those set forth in the Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Köster U.S. Patents Nos. 4,725,677 and Re. 34,069. In general the alkyl or cyanoethyl withdrawing groups are preferred, as commercially available phosphoramidites generally incorporate either the methyl or cyanoethyl phosphorus protecting group.

The method for removal of phosphorus protecting groups (pg's) depends upon the specific pg to be removed. The β-eliminable groups, such as those disclosed in the Köster et al. patents, are generally removed in a weak base solution, whereby an acidic β-hydrogen is extracted and the $-CH_2CH_2-G_w$ group is eliminated by rearrangement to form the corresponding acrylo-compound $CH_2=CH-G_w$. In contrast, an alkyl group is generally removed by nucleophilic attack on the α-carbon of the alkyl group. Such pg's are described in the Caruthers et al. patents, as cited herein.

The person skilled in the art will recognize that oxidation of P(III) to P(V) can be carried out by a variety of reagents. Furthermore, the person skilled in the art will recognize that the P(V) species can exist as phosphate triesters, phosphorothioate diesters, or phosphorodithioate diesters. Each type of P(V) linkage has uses and advantages, as described herein. Thus, the term "oxidizing agent" should be understood broadly as being any reagent capable of transforming a P(III) species (e.g. a phosphite) into a P(V) species. Thus the term "oxidizing agent" includes "sulfurizing agent," and oxidation will be understood to embrace both introduction of oxygen and introduction of sulfur, or sulfurization. Where it is important to indicate that an oxidizing agent introduces an oxygen into a P(III) species to make a P(V) species, the oxidizing agent will be referred to herein is "an oxygen-introducing oxidizing reagent."

Oxidizing reagents for making phosphate diester linkages (i.e. oxygen-introducing oxidizing reagents) under the phosphoramidite protocol have been described by e.g. Caruthers et al. and Köster et al., as cited herein. Examples of sulfurization reagents which have been used to synthesize oligonucleotides containing phosphorothioate bonds include elemental sulfur, dibenzoyltetrasulfide, 3-H-1,2-benzidithiol-3-one 1,1-dioxide (also known as Beaucage reagent), tetraethylthiuram disulfide (TETD), and bis-(O,O-diisopropoxy phosphinothioyl) disulfide (known as Stec reagent). Oxidizing reagents for making phosphorothioate diester linkages include phenyl acetyl disulfide (PADS), as described by Cole et al. in U.S. Patent No. 6,242,591. In some embodiments of the invention, the phosphorothioate diester and phosphate diester linkages may alternate between sugar subunits. In other embodiments of the present invention, phosphorothioate linkages alone may be employed.

Various solvents may be used in the oxidation reaction. Suitable solvents are identified in the Caruthers et al. and Köster et al. patents, cited herein. The Cole et al. patent describes acetonitrile as a solvent for phenyl acetyl disulfide. Other suitable solvents include toluene, xanthenes, dichloromethane, etc.

Reagents for cleaving an oligonucleotide from a support are set forth, for example, in the Caruthers et al. and Köster et al. patents, as cited herein.

The oligonucleotide may be worked up by standard procedures known in the art, for example by size exclusion chromatography, high performance liquid chromatography (e.g. reverse-phase HPLC), differential precipitation, etc. In some embodiments according to the present invention, the oligonucleotide is cleaved from a solid support while the 5'-OH protecting group is still on the ultimate nucleoside. This so-called DMT-on (or trityl-on) oligonucleotide is then subjected to chromatography, after which the DMT group is removed by treatment in an organic acid, after which the oligonucleotide is de-salted and further purified to form a final product.

The 5'-hydroxyl protecting groups may be any groups that are selectively removed under suitable conditions. In particular, the 4,4'-dimethoxytriphenylmethyl (DMT) group is a favored group for protecting at the 5'-position, because it is readily cleaved under acidic conditions (e.g. in the presence of dichloroacetic acid (DCA), trichloroacetic acid (TCA), or acetic acid. Removal of DMT

from the support-bound oligonucleotide is generally performed with DCA. Removal of oligonucleotide after cleavage from the support is generally performed with acetic acid.

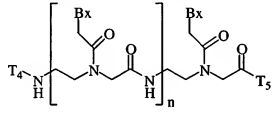
As described herein, oligonucleotides can be prepared as chimeras with other oligomeric moieties. In the context of this invention, the term "oligomeric compound" refers to a polymeric structure capable of hybridizing a region of a nucleic acid molecule, and an "oligomeric moiety" a portion of such an oligomeric compounds. Oligomeric compounds include oligonucleotides, oligonucleosides, oligonucleotide analogs, modified oligonucleotides and oligonucleotide mimetics. Oligomeric compounds can be linear or circular, and may include branching. They can be single stranded or double stranded, and when double stranded, may include overhangs. In general an oligomeric compound comprises a backbone of linked monomeric subunits where each linked monomeric subunit is directly or indirectly attached to a heterocyclic base moiety. The linkages joining the monomeric subunits, the monomeric subunits and the heterocyclic base moieties can be variable in structure giving rise to a plurality of motifs for the resulting oligomeric compounds including hemimers, gapmers and chimeras. As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base moiety. The two most common classes of such heterocyclic bases are purines and pyrimidines. In the context of this invention, the term " oligonucleoside" refers to nucleosides that are joined by internucleoside linkages that do not have phosphorus atoms. Internucleoside linkages of this type include short chain alkyl, cycloalkyl, mixed heteroatom alkyl, mixed heteroatom cycloalkyl, one or more short chain heteroatomic and one or more short chain heterocyclic. These internucleoside linkages include but are not limited to siloxane, sulfide, sulfoxide, sulfone, acetyl, formacetyl, thioformacetyl, methylene formacetyl, thioformacetyl, alkeneyl, sulfamate; methyleneimino, methylenehydrazino, sulfonate, sulfonamide, amide and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In the context of this invention, the term "oligonucleotide mimetic" refers to an oligonucleotide wherein the backbone of the nucleotide units has been replaced with novel groups. Although the term is intended to include oligomeric compounds wherein only the furanose ring or both the furanose ring and the internucleotide linkage are replaced with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. Oligonucleotide mimetics can be further modified to incorporate one or more modified heterocyclic base moieties to enhance properties such as hybridization.

One oligonucleotide mimetic that has been reported to have excellent hybridization properties, is peptide nucleic acids (PNA). The backbone in PNA compounds is two or more linked aminoethylglycine units which gives PNA an amide containing backbone. The heterocyclic base moieties are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

PNA has been modified to incorporate numerous modifications since the basic PNA structure was first prepared. The basic structure is shown below:



wherein

Bx is a heterocyclic base moiety;

T_4 is hydrogen, an amino protecting group, $-C(O)R_5$, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group, a reporter group, a conjugate group, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

T_5 is -OH, $-N(Z_1)Z_2$, R_5, D or L α-amino acid linked via the α-amino group or optionally through the ω-amino group when the amino acid is lysine or ornithine or a peptide derived from D, L or mixed D and L amino acids linked through an amino group, a chemical functional group, a reporter group or a conjugate group;

Z_1 is hydrogen, C_1-C_6 alkyl, or an amino protecting group;

Z_2 is hydrogen, C_1-C_6 alkyl, an amino protecting group, $-C(=O)-(CH_2)_n-J-Z_3$, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group;

Z_3 is hydrogen, an amino protecting group, $-C_1$-C_6 alkyl, $-C(=O)-CH_3$, benzyl, benzoyl, or $-(CH_2)_n-N(H)Z_1$;

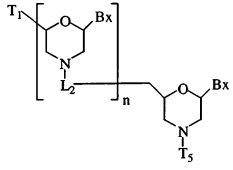
each J is O, S or NH;

R_5 is a carbonyl protecting group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acids) having heterocyclic base moieties attached to the morpholino ring. There are a number of linking groups reported that are used to link the morpholino rings. A preferred class of linking groups were selected as being non-ionic. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, Biochemistry, 2002, 41(14), 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991.

The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups (L_2) joining the monomeric subunits. The formula of the basic morpholino oligomeric compound is shown below:



wherein

T_1 is hydroxyl or a protected hydroxyl;

T_5 is hydrogen or a phosphate or phosphate derivative;

L_2 is a linking group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units having heterocyclic bases attached to the morpholino ring. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, *Biochemistry*, **2002**, *41(14)*, 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991.

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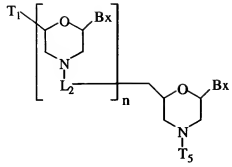


wherein

T_1 is hydroxyl or a protected hydroxyl;

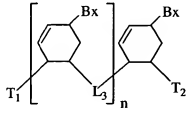
T_5 is hydrogen or a phosphate or phosphate derivative;

L_2 is a linking group; and

n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al., J. Am. Chem. Soc.*, **2000**, *122*, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed with easy conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



wherein

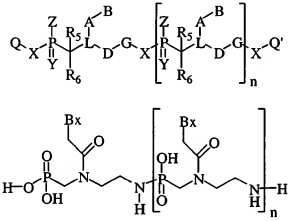
each Bx is a heterocyclic base moiety;

T_1 is hydroxyl or a protected hydroxyl; and

T_2 is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids which in some embodiments have a similarity to PNA but incorporate a phosphorus group in the backbone. This class of oligonucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting gene expression (antisense oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology. In cases where one or more of the nucleoside analogs is an LNA, the oligonucleotide mimetic may act as an LNAzyme, which may be thought of as a modified ribozyme, having the advantages of LNA, as discussed herein.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below along with one selection of Markush variables which give a compound having a resemblance to PNA.



The term "nucleobase," as used herein, is intended to by synonymous with "nucleic acid base or mimetic thereof" as herein described. In general, a nucleobase is any substructure that contains one or more atoms or groups of atoms capable of hydrogen bonding to a base of an oligonucleotide. Thus, the term "nucleobase" encompasses naturally-occurring purines and pyrimidines (guanine, adenine, thymine, cytidine and uracil), as well as protected analogs thereof and a wide variety of mimetic moieties as described herein.

As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-

substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine, 7-propynyl-7-deaza-8-azaguanine, 7-propynyl-7-deaza-8-azaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch *et al.*, *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**.

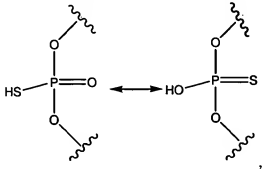
Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

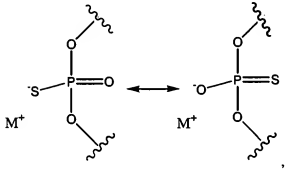
In general, the term base includes the term nucleobase as described above. The term "base" means a binding member, as described hereinabove. While nucleobases are generally heterocyclic moieties, the term "base" as used herein with means any moiety or residue capable of participating in specific binding to a naturally occurring nucleobase residue.

Oligonucleotides as defined herein generally include salts, solvates and tautomers of oligonucleotides. In general, many bases, especially nucleobases, can form tautomeric structures that

are included within the general definitions of oligonucleotides according to the present invention. In addition, the phosphorothioate linker can form the following tautomers:



, and can likewise form the following salt structures:



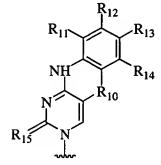
, wherein M^+ is a suitable salt-forming cation, such as Na^+, K^+, ½ Ca^{2+}, ½ Mg^{2+}, 1/3 Al^{3+}, NH_4^+, H_3O^+, etc. (The fractions indicate fractional equivalents of the cationic species per phosphate diester linkage.)

Additional modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. For example, one additional modification of the ligand conjugated oligonucleotides of the present invention involves chemically linking to the oligonucleotide one or more additional non-ligand moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 111; Kabanov et al., FEBS Lett., 1990, 259, 327; Svinarchuk et al., Biochimie, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651; Shea et al., Nucl. Acids Res., 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651), a palmityl

moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patents Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned, and each of which is herein incorporated by reference.

In some embodiments of the present invention oligomeric compounds are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications selectively bind to guanosines. Hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:

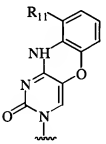


Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one (R_{10} = O, R_{11} - R_{14}= H) [Kurchavov, *et al., Nucleosides and Nucleotides*, 1997, 16, 1837-1846], 1,3-diazaphenothiazine-2-one (R_{10}= S, R_{11} - R_{14}= H), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. Am. Chem. Soc. 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one (R_{10} = O, R_{11} - R_{14} = F) [Wang, J.; Lin, K.-Y., Matteucci, M. Tetrahedron Lett. 1998, 39, 8385-8388]. Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions (also see U.S. Patent

Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this application and are herein incorporated by reference in their entirety).

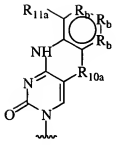
Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold (R_{10} = O, R_{11} = -O-$(CH_2)_2$-NH_2, $R_{12\text{-}14}$=H) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a ΔT_m of up to 18° relative to 5-methyl cytosine ($dC5^{me}$), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The T_m data indicate an even greater discrimination between the perfect match and mismatched sequences compared to $dC5^{me}$. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety. Such compounds include those having the formula:



Wherein R_{11} includes $(CH_3)_2N$-$(CH_2)_2$-O-; H_2N-$(CH_2)_3$-; Ph-CH_2-O-C(=O)-N(H)-$(CH_2)_3$-; H_2N-; Fluorenyl-CH_2-O-C(=O)-N(H)-$(CH_2)_3$-; Phthalimidyl-CH_2-O-C(=O)-N(H)-$(CH_2)_3$-; Ph-CH_2-O-C(=O)-N(H)-$(CH_2)_2$-O-; Ph-CH_2-O-C(=O)-N(H)-$(CH_2)_3$-O-; $(CH_3)_2N$-N(H)-$(CH_2)_2$-O-; Fluorenyl-CH_2-O-C(=O)-N(H)-$(CH_2)_2$-O-; Fluorenyl-CH_2-O-C(=O)-N(H)-$(CH_2)_3$-O-; H_2N-$(CH_2)_2$-O-CH_2-; N_3-$(CH_2)_2$-O-CH_2-; H_2N-$(CH_2)_2$-O-, and $NH_2C(=NH)NH$-.

Also disclosed are tricyclic heterocyclic compounds of the formula:



Wherein

R_{10a} is O, S or N-CH_3;

R_{11a} is $A(Z)_{x1}$,wherein A is a spacer and Z independently is a label bonding group bonding group optionally bonded to a detectable label, but R_{11a} is not amine, protected amine, nitro or cyano;

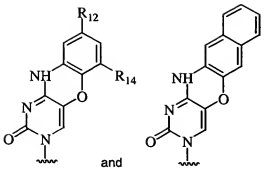
X1 is 1, 2 or 3; and

R_b is independently -CH=, -N=, -C(C_{1-8} alkyl)= or -C(halogen)=, but no adjacent R_b are both -N=, or two adjacent R_b are taken together to form a ring having the structure:

where R_c is independently -CH=, -N=, -C(C_{1-8} alkyl)= or -C(halogen)=, but no adjacent R_b are both -N=.

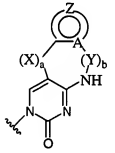
The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further tricyclic and tetracyclic heteroaryl compounds amenable to the present invention include those having the formulas:



wherein R_{14} is NO_2 or both R_{14} and R_{12} are independently $-CH_3$. The synthesis of these compounds is disclosed in United States Patent Serial Number 5,434,257, which issued on July 18, 1995, United States Patent Serial Number 5,502,177, which issued on March 26, 1996, and United States Patent Serial Number 5,646, 269, which issued on July 8, 1997, the contents of which are commonly assigned with this application and are incorporated herein in their entirety.

Further tricyclic heterocyclic compounds amenable to the present invention also disclosed in the "257, 177 and 269" Patents include those having the formula:



a and b are independently 0 or 1 with the total of a and b being 0 or 1;

A is N, C or CH;

X is S, O, C=O, NH or NCH_2, R^6;

Y is C=O;

Z is taken together with A to form an aryl or heteroaryl ring structure comprising 5 or 6 ring atoms wherein the heteroaryl ring comprises a single O ring heteroatom, a single N ring heteroatom, a single S ring heteroatom, a single O and a single N ring heteroatom separated by a carbon atom, a single S and a single N ring heteroatom separated by a C atom, 2 N ring heteroatoms separated by a carbon atom, or 3 N ring heteroatoms at least 2 of which are separated by a carbon atom, and wherein the aryl or heteroaryl ring carbon atoms are unsubstituted with other than H or at least 1 non-bridging ring carbon atom is substituted with R^{20} or =O;

or Z is taken together with A to form an aryl ring structure comprising 6 ring atoms wherein the aryl ring carbon atoms are unsubstituted with other than H or at least 1 non-bridging ring carbon atom is substituted with R^6 or =O;

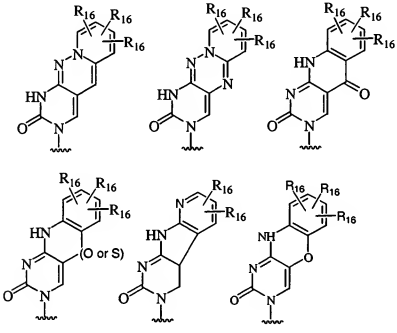
R^6 is independently H, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, NO_2, $N(R^3)_2$, CN or halo, or an R^6 is taken together with an adjacent Z group R^6 to complete a phenyl ring;

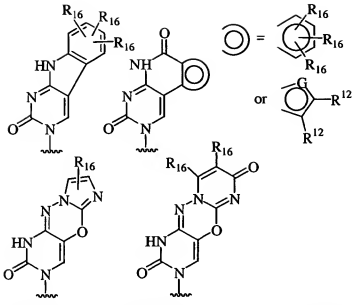
R^{20} is , independently, H, C_{1-6} alkyl, C_{2-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, NO_2, $N(R^{21})_2$, CN, or halo, or an R^{20} is taken together with an adjacent R^{20} to complete a ring containing 5 or 6 ring atoms, and tautomers, solvates and salts thereof;

R^{21} is, independently, H or a protecting group;

R^3 is a protecting group or H; and tautomers, solvates and salts thereof.

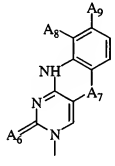
More specific examples included in the "257, 177 and 269" Patents are compounds of the formula:





wherein each R_{16}, is, independently, selected from hydrogen and various substituent groups.

Further polycyclic base moieties having the formula:

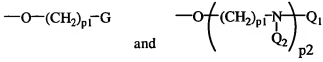


wherein:

A_6 is O or S;

A_7 is CH_2, N-CH_3, O or S;

each A_8 and A_9 is hydrogen or one of A_8 and A_9 is hydrogen and the other of A_8 and A_9 is selected from the group consisting of:



wherein:

wherein:

G is -CN, -OA_{10}, -SA_{10}, -$N(H)A_{10}$, -$ON(H)A_{10}$ or -$C(=NH)N(H)A_{10}$;

Q_1 is H, -NHA_{10}, -$C(=O)N(H)A_{10}$, -$C(=S)N(H)A_{10}$ or -$C(=NH)N(H)A_{10}$;

each Q_2 is, independently, H or Pg;

A_{10} is H, Pg, substituted or unsubstituted C_1-C_{10} alkyl, acetyl, benzyl, $-(CH_2)_{p3}NH_2$, $-(CH_2)_{p3}N(H)Pg$, a D or L α-amino acid, or a peptide derived from D, L or racemic α-amino acids;

Pg is a nitrogen, oxygen or thiol protecting group;

each p1 is, independently, from 2 to about 6;

p2 is from 1 to about 3; and

p3 is from 1 to about 4;

are disclosed in Unites States Patent Application Serial number 09/996,292 filed November 28, 2001, which is commonly owned with the instant application, and is herein incorporated by reference.

Exemplary preferred antisense compounds include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly preferred antisense compounds are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the empirically-derived preferred antisense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense compounds.

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are herein identified as preferred embodiments of the invention. While specific sequences of the antisense compounds are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional preferred antisense compounds may be identified by one having ordinary skill.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides

that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000,** 480, 17-24; Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16), SAGE (serial analysis of gene expression)(Madden, *et al.*, *Drug Discov. Today,* **2000,** 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999,** 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, **2000,** 97, 1976-81), protein arrays and proteomics (Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16; Jungblut, *et al.*, *Electrophoresis,* **1999,** 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16; Larsson, *et al.*, *J. Biotechnol.*, **2000,** 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, *et al.*, *Anal. Biochem.*, **2000,** 286, 91-98; Larson, *et al.*, *Cytometry,* **2000,** 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000,** 3, 316-21), comparative genomic hybridization (Carulli, *et al.*, *J. Cell Biochem. Suppl.*, **1998,** 31, 286-96), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer,* **1999,** 35, 1895-904) and mass spectrometry methods (reviewed in To, *Comb. Chem. High Throughput Screen,* **2000,** 3, 235-41).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities

that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding a particular protein. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding a particular protein, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation

codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It has also been found that introns can be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and extronic regions.

Upon excision of one or more exon or intron regions or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable.

An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed. It is preferred that the antisense compounds of the present invention comprise at least 80% sequence complementarity with the target nucleic acid, more that they comprise 90% sequence complementarity and even more comprise 95% sequence complementarity with the target nucleic acid sequence to which they are targeted. Percent complementarity of an antisense compound with a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul et al., *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656).

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The sites to which these preferred antisense compounds are specifically hybridizable are hereinbelow referred to as "preferred target regions" and are therefore preferred sites for targeting. As used herein the term "preferred target region" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently

believed that these target regions represent regions of the target nucleic acid which are accessible for hybridization.

While the specific sequences of particular preferred target regions are set forth below, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional preferred target regions may be identified by one having ordinary skill.

Target regions 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative preferred target regions are considered to be suitable preferred target regions as well.

Exemplary good preferred target regions include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly good preferred target regions are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the empirically-derived preferred target regions illustrated herein will be able, without undue experimentation, to identify further preferred target regions. In addition, one having ordinary skill in the art will also be able to identify additional compounds, including oligonucleotide probes and primers, that specifically hybridize to these preferred target regions using techniques available to the ordinary practitioner in the art.

The ability of oligonucleotides to bind to their complementary target strands is compared by determining the melting temperature (T_m) of the hybridization complex of the oligonucleotide and its complementary strand. The melting temperature (T_m), a characteristic physical property of double helices, denotes the temperature (in degrees centigrade) at which 50% helical (hybridized) versus coil (unhybridized) forms are present. T_m is measured by using the UV spectrum to determine the formation and breakdown (melting) of the hybridization complex. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher T_m. The higher the T_m, the greater the strength of the bonds between the strands. The structure-stability relationships of a large number of nucleic acid modifications have been reviewed (Freier and Altmann, Nucl. Acids Research, 1997, 25, 4429-443).

EXAMPLES

The present invention may be further appreciated upon reference to the following, non-limiting examples.

Examples

2,7-Dimethy1-9-phenylxanthen-9-ol (DMPx-OH)

Tolyl ether (20 g, 0.10 mol), α,α,α-trichlorotoluene (20 ml, 0.12 mol), zinc chloride (40 g, 0.29 mol) and phosphorus oxychloride (30 ml, 0.32 mol) were stirred in an oil bath (84°C) for 1 hour. The mixture was cooled to room temperature and poured into water (500 ml). The flask was rinsed with ethyl acetate (50 ml) and the suspension was stirred overnight. The mixture was then filtered, washed with water and methanol and dried to give the crude title compound as a solid (34.41 g).

2,7-Dimethyl-9-phenylxanthen-9-ol (DMPx-OH)

Tolyl ether (10 g), benzoic acid (7.5 g), zinc chloride (20 g) and phosphorus oxychloride (15 ml) were stirred in an oil bath (95°C) for two hours. The mixture was cooled to room temperature and ethyl acetate (25 ml) was added to form a suspension.

The suspension was poured into 500 ml stirring DI water at room temperature. The mixture was heated under reflux for 15 minutes and cooled down to room temperature overnight. The mixture was filtered and washed with water (100ml). The damp cake was suspended with 300 ml of methanol and stirred to boil for 2 or 3 minutes. The resultant suspension was allowed to cool to room temperature over a period of 3 hrs and was then filtered, washed with methanol and dried to give the title compound as a solid (14 g, 91.8%).

9-Chloro-2,7-Dimethyl-9-phenylxanthene (DMPx-Cl)

DMPx (DMPx-OH) was dissolved in methylene chloride (10 ml), and acetyl chloride (1 ml) was added. The mixture was stirred at room temperature for 15 min and evaporated under reduce pressure to a solid. The residue was stirred with n-hexane (200 ml) at room temperature. The solid was filtered and washed with n-hexane to give the title product.

2,7-Bromo-9-phenylxanthen-9-01 (DMPx-OH)

Bis-(4-bromophenyl) ether (30 g, 0.092 mol), α,α,α-trichlorotoluene (22 ml, 0.15 mol), aluminum chloride (20 g, 0.15 mol) in dichloromethane (75 ml) were stirred at room temperature for 1 hour. The reaction mixture was poured into water (100 ml) and hexane (300 ml and the suspension was stirred overnight. The mixture was then filtered, washed with water (230 ml) and hexane 400 ml) and dried to give the title compound as a crystalline solid (34.94 g, yield: 88%).

5'-DMPx-thymidine

Thymidine (2.4 g, 10 mmol) was dissolved in pyridine (15 ml) and DMPx-Cl (4.1 g, 11.5 mmol) was added. The mixture was stirred at room temperature for 30 min. The mixture was diluted with ethyl acetate (50 ml) and washed with water (2x50 ml). The mixture was evaporated to dryness and the solid was dissolved in dichloromethane (15 ml). Hexane (50 ml) was added and the mixture was stirred overnight. Filtration gave the title compound as a solid (4.44 g, 79%).

The person having skill in the art will recognize that further embodiments are possible within the general scope of the foregoing description and the attached drawings and claims, and it would be within the skill of such skilled person to practice the invention as generally described herein.

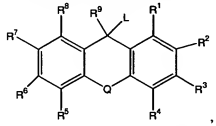
All references cited herein are expressly incorporated herein by reference.

ABSTRACT OF THE DISCLOSURE

A new process of making pixylating reagents avoids the use of Grignard reagents. The new process includes a Friedel-Crafts reaction, which permits manufacture of a variety of protecting groups having various properties. Novel reagents, protected nucleosides, amidites and oligonucleotides are also provided. The novel protecting groups may be made more or less acid labile, depending upon the specific substituents on the xanthyl and phenyl rings of the protecting group. Thus in some embodiments, the groups may be removed in weaker acids than heretofore employed, and may in some cases be used as groups orthogonal to protecting groups that are labile only to stronger acids.

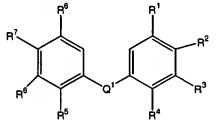
We Claim:

1. A process of manufacturing a product compound of the formula:



wherein R^1-R^8 are each, independently, H, alkyl, aryl, alkenyl, alkynyl, cyano, azido, halo, hydroxy, -C(=O)O-alkyl, -O-C(=O)-R^{10}, -C(=O)N(R^{10})R^{11}, -NHC(=O)R^{10}, -N(R^{10})R^{11}, -O-R^{12}, -S-R^{12}, or two or more groups R^1-R^8, together with the ring carbons to which they are bonded, combine to form a fused ring; R^9 is alkyl, alkenyl, alkynyl or aryl, wherein aryl may be substituted with one or more groups as defined by R^1; R^{10} is H or C_1-C_{24} alkyl, R^{11} is H or C_1-C_{24} alkyl, and R^{12} is C_1-C_{24} alkyl; and L is a leaving group; and Q is O, S, N(C=O)-R^{10}, or NR^{10};

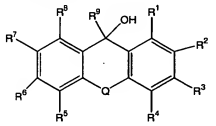
said process comprising reacting a first compound of the formula:



with a second compound of the formula:

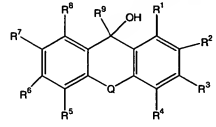
wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, C(=O)OR^{13}, or C(=O)N$R^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{24} alkyl; in the presence of a Friedel-Crafts acid;

to produce an intermediate compound of the formula:

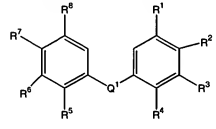


and reacting the intermediate compound with a reagent for introducing the leaving group L to form said product compound.

2. A process of manufacturing a product compound of the formula:



said process comprising reacting a first compound of the formula:



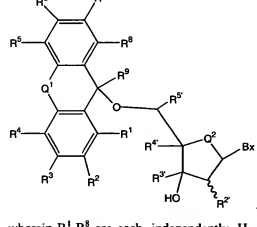
with a second compound of the formula:


,

wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, $C(=O)OR^{13}$, or $C(=O)NR^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{24} alkyl; in the presence of a Friedel-Crafts acid;

to produce said product compound.

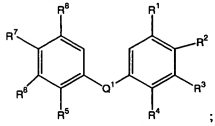
3. A process of manufacturing a protected nucleoside having the formula:


,

wherein R^1-R^8 are each, independently, H, alkyl, aryl, alkenyl, alkynyl, cyano, azido, halo, hydroxy, -C(=O)O-alkyl, -O-C(=O)-R^{10}, -C(=O)N(R^{10})R^{11}, -NHC(=O)R^{10}, -N(R^{10})R^{11}, -O-

R^{12}, -S-R^{12}, or two or more groups R^1-R^8, together with the ring carbons to which they are bonded, combine to form a fused ring; R^9 is alkyl, alkenyl, alkynyl or aryl, wherein aryl may be substituted with one or more groups as defined by R^1; R^{10} is H or C_1-C_{24} alkyl, R^{11} is H or C_1-C_{24} alkyl, and R^{12} is C_1-C_{24} alkyl, Q^1 is O, S, NR^{10} or $NC(=O)R^{10}$, Q^2 is Q^1, -CH_2-, or –CH=CH-; $R^{2'}$ is H, OH, reversibly protected OH, or a 2'-substituent; $R^{3'}$ is H, OH or reversibly protected OH, $R^{4'}$ is H, a substituent or together with $R^{2'}$ forms a bridge, and $R^{5'}$ is H or together with $R^{4'}$ form a bridge, which may be fused to a further ring or ring system; and Bx is a nucleobase;

said process comprising reacting a first compound of the formula:



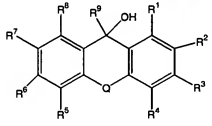
;

with a second compound of the formula:

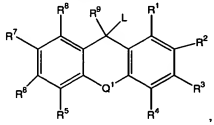
,

wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, $C(=O)OR^{13}$, or $C(=O)NR^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{24} alkyl; in the presence of a Friedel-Crafts acid;

to produce an intermediate compound of the formula:



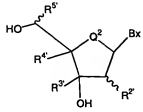
reacting the intermediate compound with a reagent for introducing the leaving group L to form a reagent compound of formula:



,

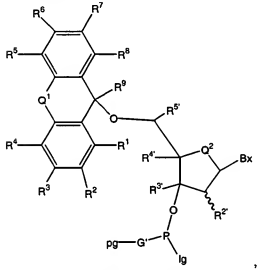
wherein L is a leaving group;

and contacting the reagent compound with a nucleoside of formula:



to form the protected nucleoside.

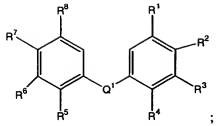
4. A method of making a protected amidite of having the formula:



,

wherein R^1-R^8 are each, independently, H, alkyl, aryl, alkenyl, alkynyl, cyano, azido, halo, hydroxy, -C(=O)O-alkyl, -O-C(=O)-R^{10}, -C(=O)N(R^{10})R^{11}, -NHC(=O)R^{10}, -N(R^{10})R^{11}, -O-R^{12}, -S-R^{12}, or two or more groups R^1-R^8, together with the ring carbons to which they are bonded, combine to form a fused ring; R^9 is alkyl, alkenyl, alkynyl or aryl, wherein aryl may be substituted with one or more groups as defined by R^1; R^{10} is H or C_1-C_{24} alkyl, R^{11} is H or C_1-C_{24} alkyl, and R^{12} is C_1-C_{24} alkyl, Q^1 is O, S, NR^{10} or NC(=O)R^{10}, Q^2 is Q^1, -CH_2-, or –CH=CH-; $R^{2'}$ is H, OH, reversibly protected OH, or a 2'-substituent; $R^{3'}$ is H, OH or reversibly protected OH, $R^{4'}$ is H, a substituent or together with $R^{2'}$ forms a bridge, and $R^{5'}$ is H or together with $R^{4'}$ form a bridge, which may be fused to a further ring or ring system; and Bx is a nucleobase; pg is a phosphorus protecting group, G' is O or S, and lg is a leaving group;

said process comprising reacting a first compound of the formula:



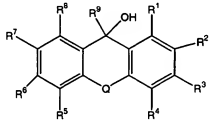
;

with a second compound of the formula:

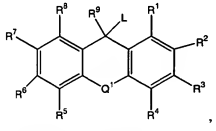
,

wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, $C(=O)OR^{13}$, or $C(=O)NR^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{18} alkyl; in the presence of a Friedel-Crafts acid;

to produce an intermediate compound of the formula:



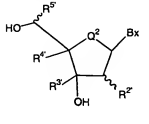
reacting the intermediate compound with a reagent for introducing the leaving group L to form a reagent compound of formula:



,

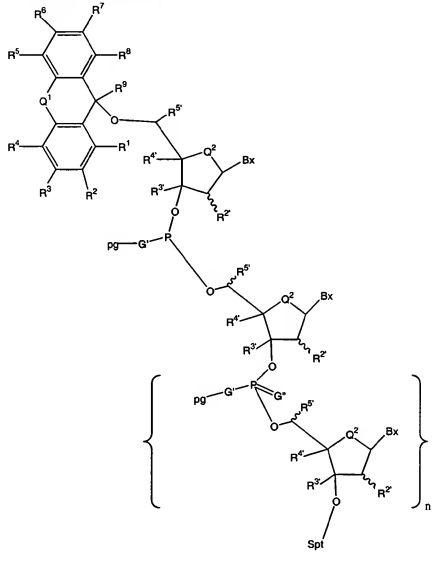
wherein L is a leaving group;

contacting the reagent compound with a nucleoside of formula:

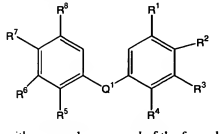


and reacting the protected nucleoside with a phosphitylating agent to produce said protected amidite.

5. A method of making a support-bound compound of formula:



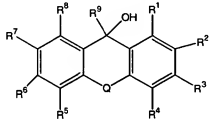
the method comprising reacting a first compound of the formula:



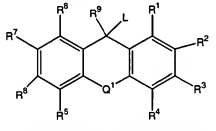
with a second compound of the formula:

wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, $C(=O)OR^{13}$, or $C(=O)NR^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{24} alkyl; in the presence of a Friedel-Crafts acid;

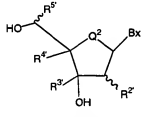
to produce an intermediate compound of the formula:



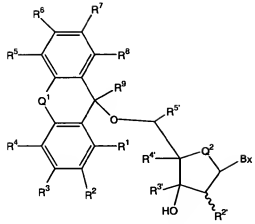
reacting the intermediate compound with a reagent for introducing the leaving group L to form a pixylating reagent of formula:



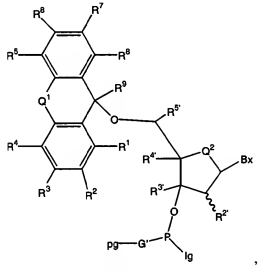
contacting the reagent compound with a nucleoside of formula:



to form a protected nucleoside of the formula:

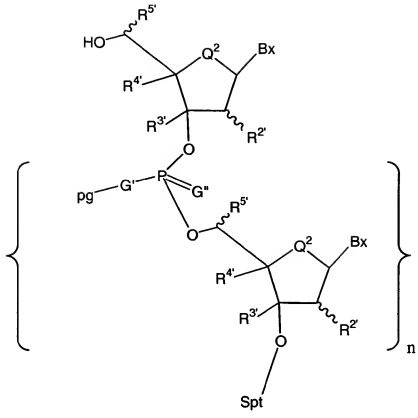


reacting the protected nucleoside with a phosphitylating agent to produce a protected amidite of formula:



wherein G' is O or S, pg is a phosphorus protecting group and lg is a leaving group;

reacting the protected amidite with a support bound intermediate of formula:

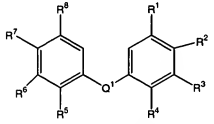


to produce a phosphite intermediate; and

oxidizing the phosphite intermediate to form the support-bound compound.

6. A method of making a support-bound oligonucleotide having the formula:

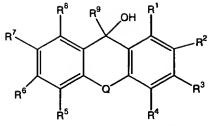
the method comprising reacting a first compound of the formula:



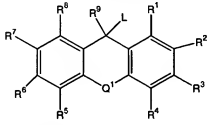
with a second compound of the formula:

wherein X^1, X^2 and X^3 are each independently halogen, or together with the carbon to which they are attached form a group selected from COOH, COX^4, $C(=O)OR^{13}$, or $C(=O)NR^{13}R^{14}$, wherein X^4 is selected from Cl, Br and I; and each of R^{13} and R^{14} is independently H or C_1-C_{24} alkyl; in the presence of a Friedel-Crafts acid;

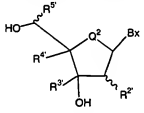
to produce an intermediate compound of the formula:



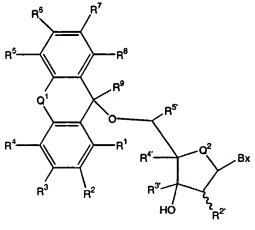
reacting the intermediate compound with a reagent for introducing the leaving group L to form a reagent compound of formula:



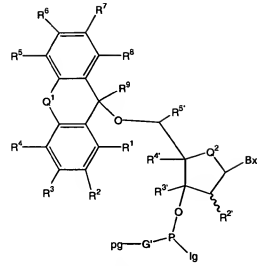
contacting the reagent compound with a nucleoside of formula:



to form a protected nucleoside of the formula:



reacting the protected nucleoside with a phosphitylating agent to produce a protected amidite of formula:



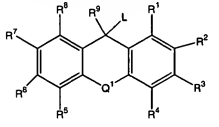
reacting the protected amidite with a support bound intermediate of formula:

to produce a phoshphite intermediate;

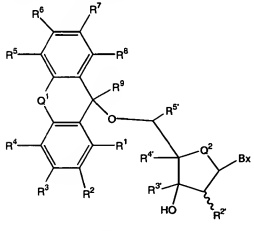
oxidizing the phosphite intermediate to form a support-bound compound of formula:

and removing the Pxl group to produce the support-bound oligonucleotide.

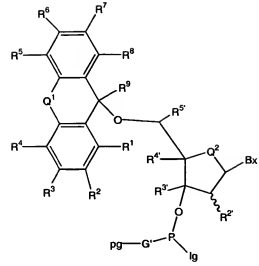
7. A pixylating reagent having the formula:



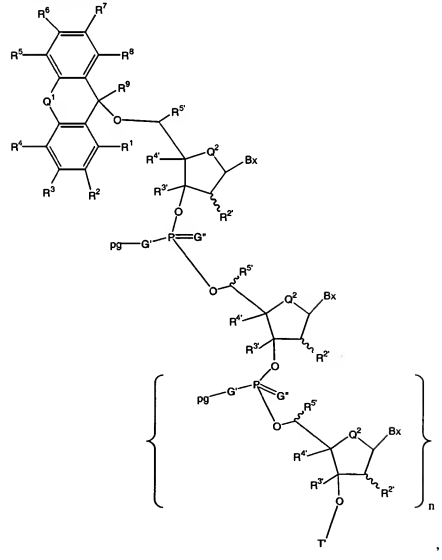
8. A protected nucleoside having the formula:



9. An amidite of formula:

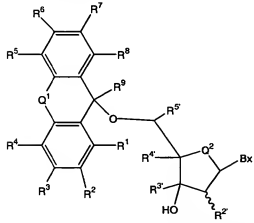


10. An oligonucleotide of formula:

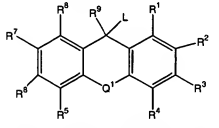


wherein T' is H, n is an integer,

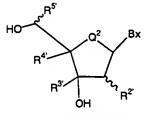
11. A process of manufacturing a protected nucleoside having the formula:



comprising reacting a pixylating reagent having the formula:

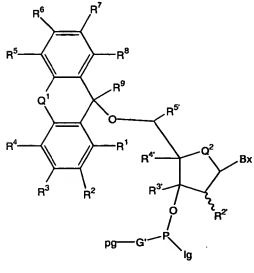


with a nucleoside having the formula:

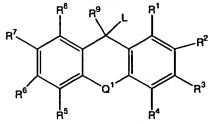


to form said protected nucleoside.

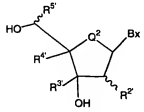
12. A process of manufacturing an amidite of formula:



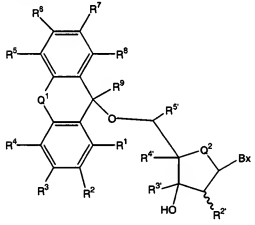
comprising reacting a pixylating reagent having formula:



with a nucleoside of formula:

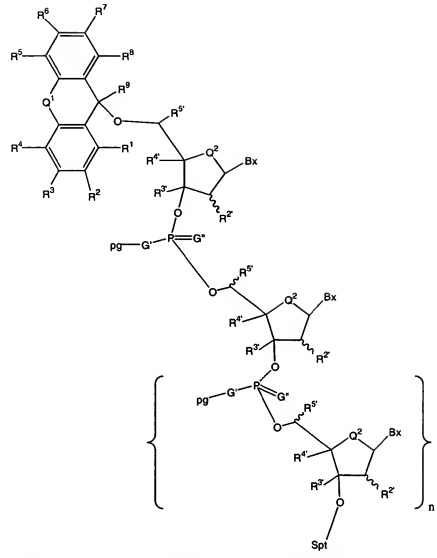


to form a protected nucleoside:

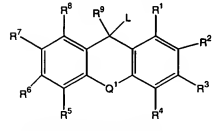


and reacting the protected nucleoside with a phosphitylating reagent to form said amidite.

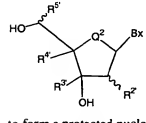
13. A process of making a support-bound compound of formula:



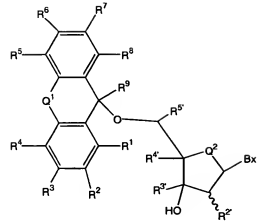
the method comprising reacting a pixylating reagent of formula:



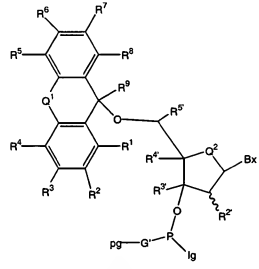
with a nucleoside of formula:



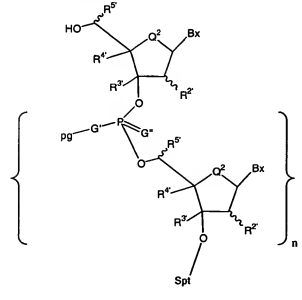
to form a protected nucleoside of the formula:



reacting the protected nucleoside with a phosphitylating agent to produce a protected amidite of formula:



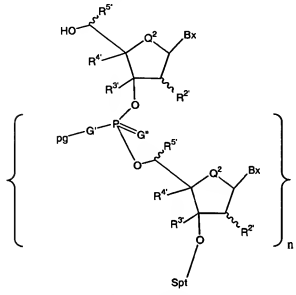
reacting the protected amidite with a support bound intermediate of formula:



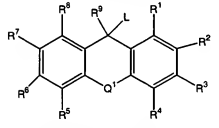
to produce a phoshphite intermediate; and

oxidizing the phosphite intermediate to form the support-bound compound.

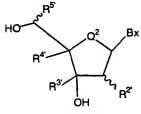
14. A method of making a support-bound oligonucleotide having the formula:



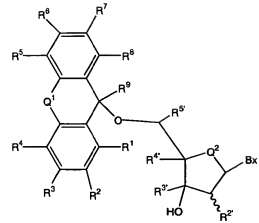
the method comprising reacting a reagent compound of formula:



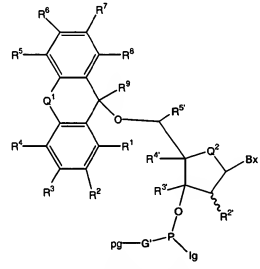
with a nucleoside of formula:



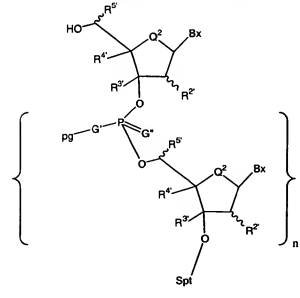
to form a protected nucleoside of the formula:



reacting the protected nucleoside with a phosphitylating agent to produce a protected amidite of formula:



reacting the protected amidite with a support bound intermediate of formula:

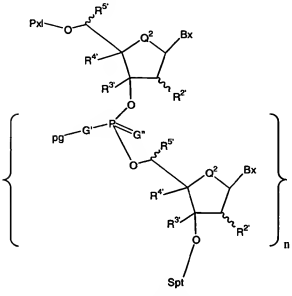


to produce a phosphite intermediate;

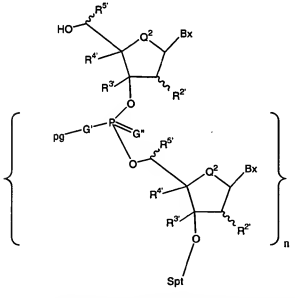
oxidizing the phosphite intermediate to form a support-bound compound of formula:

and removing the Pxl group to produce the support-bound oligonucleotide.

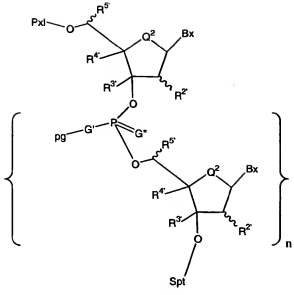
15. A method of deprotecting a protected oligonucleotide of formula:



the method comprising contacting the protected oligonucleotide with a deprotecting agent to remove the Pxl group, thereby producing a compound of formula:



16. A method of deprotecting a protected oligonucleotide of formula:

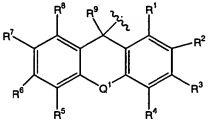


the method comprising determining a half-life for deprotection of the protected oligonucleotide in the presence of a selected deprotecting agent; and

exposing the protected oligonucleotide to the selected deprotecting agent for about 5 to about 20 half-lives.

17. A method of purifying an oligonucleotide, said method comprising:

providing a protected oligonucleotide having a protecting group:



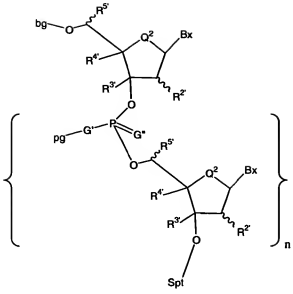
on the terminal 5'-*O* of the oligonucleotide;

subjecting the protected oligonucleotide to reverse phase liquid chromatography; and

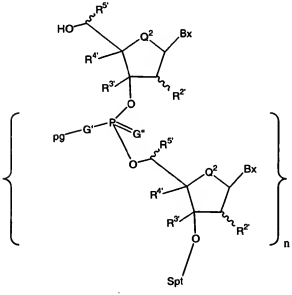
removing the protecting group.

18. A synthetic method for manufacturing an oligonucleotide, said method comprising:

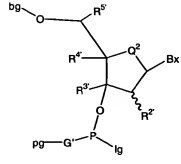
(a) providing a support-bound synthon:



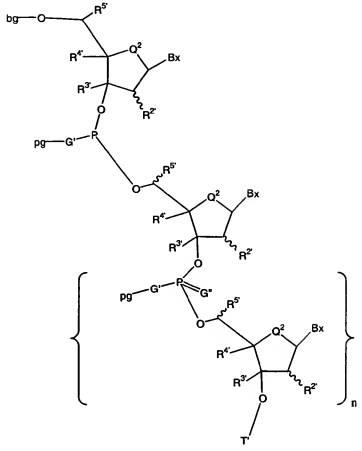
(b) contacting the support-bound synthon with a deprotecting reagent to produce the deprotected intermediate:



(c) reacting the deprotected intermediate with a phosphitylating agent of formula:



to form a phosphite intermediate:

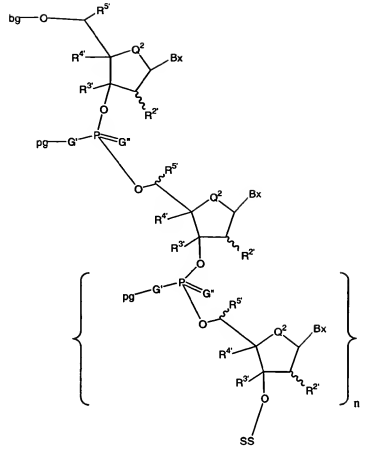


(d) in any order or simultaneously:

i. oxidizing the phosphite intermediate; and

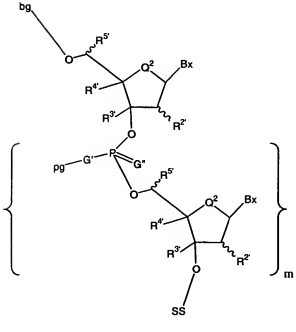
ii. capping unreacted deprotected intermediate;

to produce an extended synthon of formula:

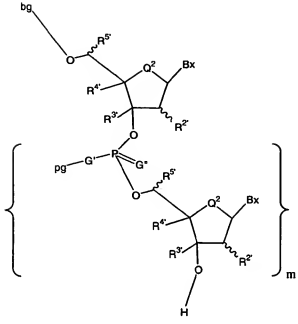


and

(e) repeating steps (a)-(e) a sufficient number of times to produce a support-bound compound of formula:



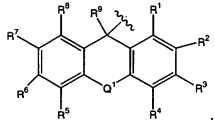
(f) cleaving the support-bound compound from the support to form a protected nucleotide:



; and

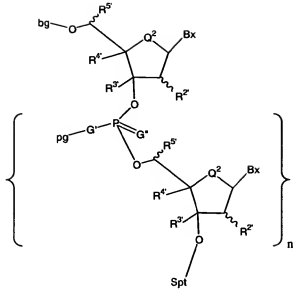
(g) removing bg from the protected nucleotide to make the oligonucleotide product,

wherein at least one of the groups represented by bg is a group Pxl:

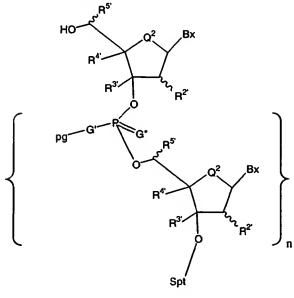


19. A synthetic method for manufacturing an oligonucleotide, said method comprising:

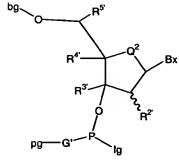
(a) providing a support-bound synthon:



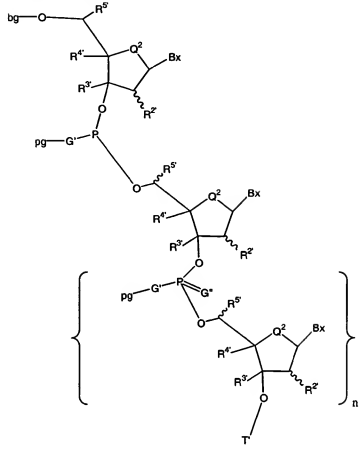
(b) contacting the support-bound synthon with a deprotecting reagent to produce the deprotected intermediate:



(c) reacting the deprotected intermediate with a phosphitylating agent of formula:



to form a phosphite intermediate:

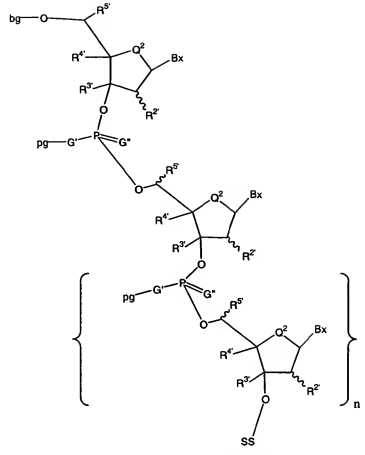


(d) in any order or simultaneously:

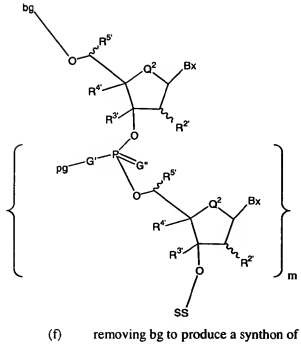
i. oxidizing the phosphite intermediate; and

ii. capping unreacted deprotected intermediate;

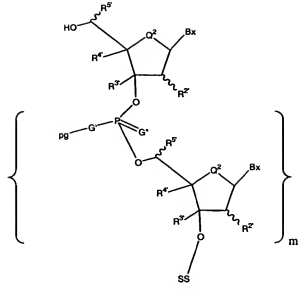
to produce an extended synthon of formula:



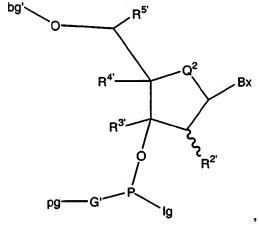
(e) repeating steps (a)-(e) a sufficient number of times to produce a support-bound compound of formula:



(f) removing bg to produce a synthon of the formula:

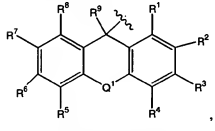


(g) reacting the synthon with a phosphitylating reagent:



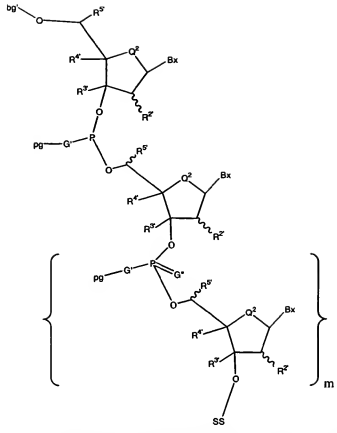
,

wherein bg' is PxI:

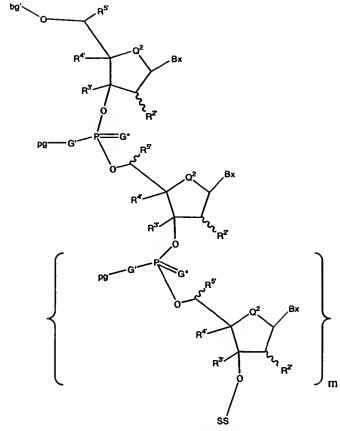


,

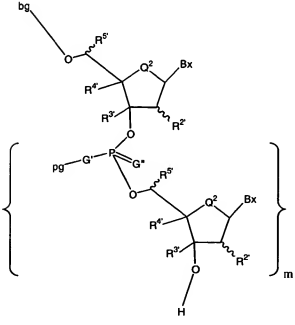
to form a phosphitylated synthon:



(h) oxidizing the phosphitylated synthon to form a compound of the formula:



(i) cleaving the support-bound compound from the support to form a protected nucleotide:



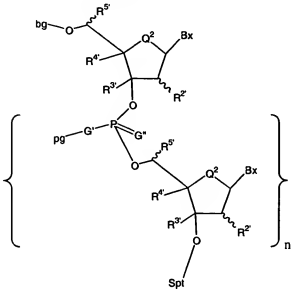
; and

(j) removing bg' from the protected nucleotide to make the oligonucleotide product.

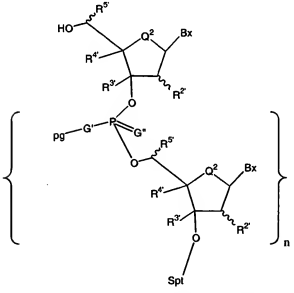
20. A synthetic method for manufacturing an oligonucleotide, said method comprising:

21. comprising:

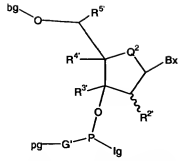
(a) providing a support-bound synthon:



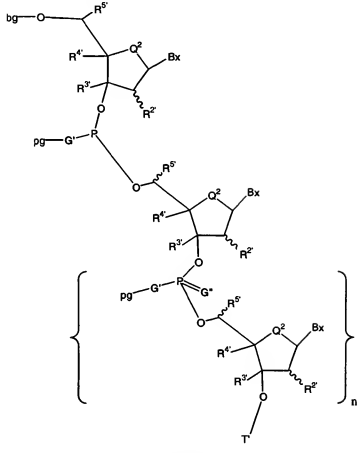
(b) contacting the support-bound synthon with a deprotecting reagent to produce the deprotected intermediate:



(c) reacting the deprotected intermediate with a phosphitylating agent of formula:



to form a phosphite intermediate:

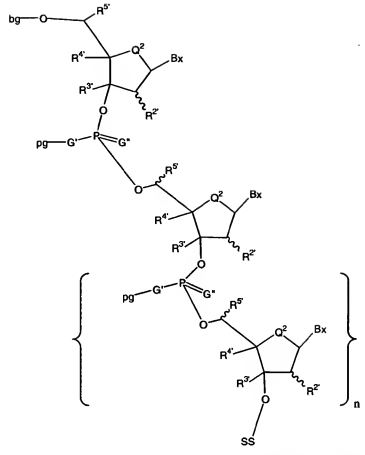


(d) in any order or simultaneously:

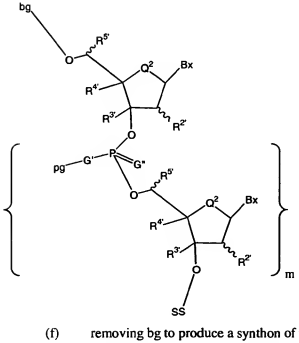
i. oxidizing the phosphite intermediate; and

ii. capping unreacted deprotected intermediate;

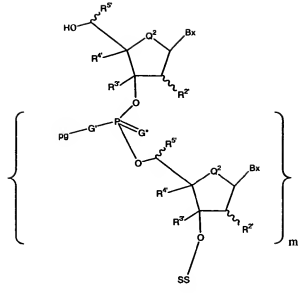
to produce an extended synthon of formula:



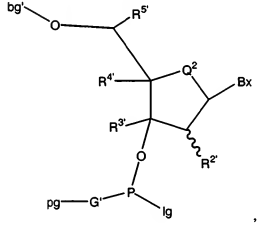
(e) repeating steps (a)-(e) a sufficient number of times to produce a support-bound compound of formula:



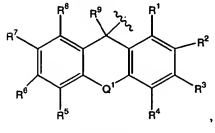
(f) removing bg to produce a synthon of the formula:



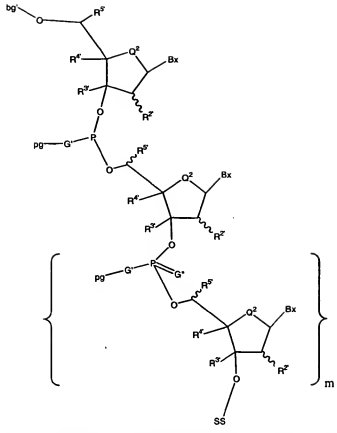
(g) reacting the synthon with a phosphitylating reagent:



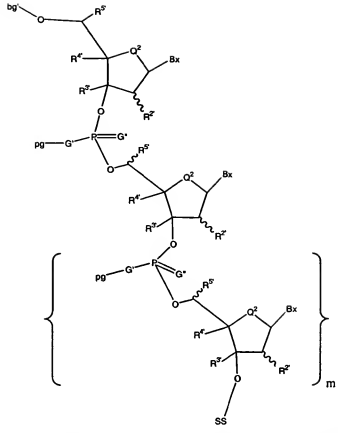
wherein bg' is PxI:



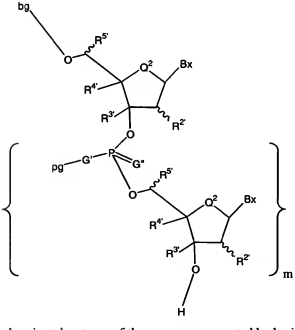
to form a phosphitylated synthon:



(h) oxidizing the phosphitylated synthon to form a compound of the formula:



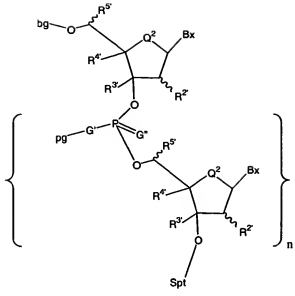
(i) cleaving the support-bound compound from the support to form a protected nucleotide:



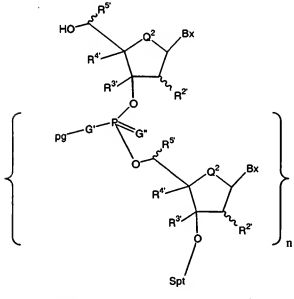
wherein at least one of the groups represented by bg is a group Pxl and bg' is Pxl.

22. A synthetic method for manufacturing an oligonucleotide, said method comprising:

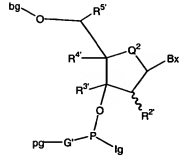
(a) providing a support-bound synthon:



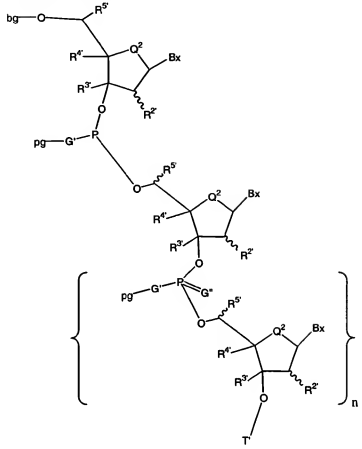
(b) contacting the support-bound synthon with a deprotecting reagent to produce the deprotected intermediate:



(c) reacting the deprotected intermediate with a phosphitylating agent of formula:



to form a phosphite intermediate:

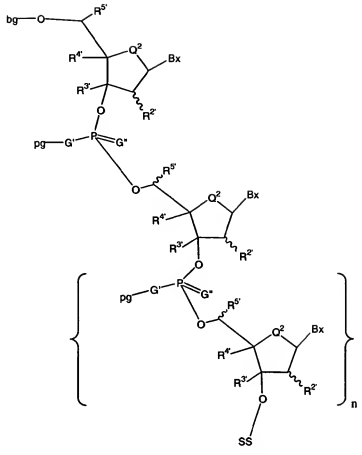


(d) in any order or simultaneously:

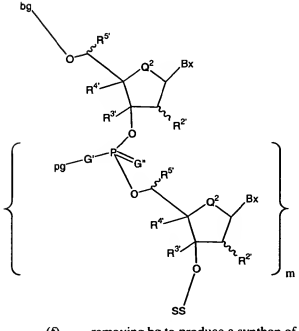
i. oxidizing the phosphite intermediate; and

ii. capping unreacted deprotected intermediate;

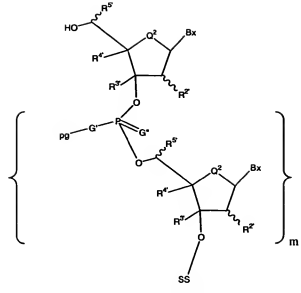
to produce an extended synthon of formula:



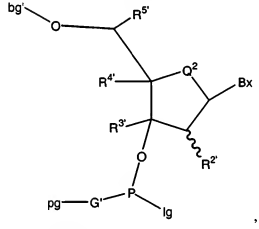
(e) repeating steps (a)-(e) a sufficient number of times to produce a support-bound compound of formula:



(f) removing bg to produce a synthon of the formula:

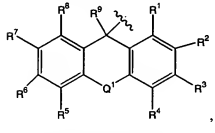


(g) reacting the synthon with a phosphitylating reagent:



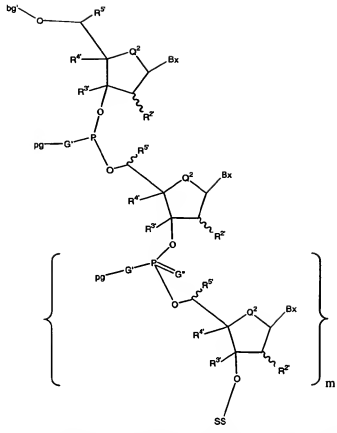
,

wherein bg' is Pxl:

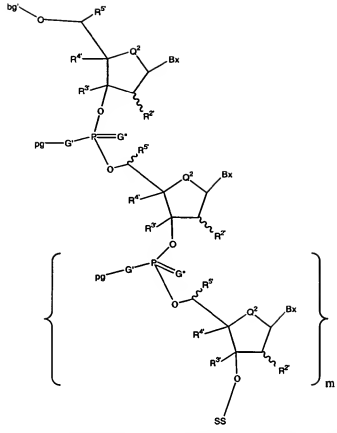


,

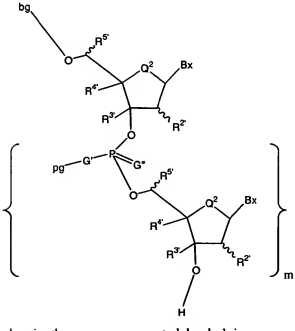
to form a phosphitylated synthon:



(h) oxidizing the phosphitylated synthon to form a compound of the formula:



(i) cleaving the support-bound compound from the support to form a protected nucleotide:



wherein the group represented by bg' is a group PxI and Bx' is uridyl or 5'-methyluridyl.